Volume 56 ▪ Number 1 ▪ March 2009

FOUNDED IN 1954

Acta Microbiologica et Immunologica Hungarica

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Acta Microbiologica et Immunologica Hungarica

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

The journal is devoted to the publication of research on medical and veterinary bacteriology, virology, mycology, parasitology, as well as on basic and clinical immunology, epidemiology, agricultural and industrial microbiology, molecular biology and biotechnology.
It is published in yearly volumes of four issues.

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Abstracted/indexed in

Biological Abstracts, BIOSIS Previews, CAB Abstracts, Chemical Abstracts, Global Health, Index Medicus, Index Veterinarius, Medline, Referativnyi Zhurnal, Science Citation Index Expanded, SCOPUS.

■

Editorial correspondence

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Subscription price

for Volume 56 (2009) in 4 issues EUR 368 + VAT (for North America: USD 516) including online access and normal postage; airmail delivery EUR 20 (USD 28).

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Publisher and distributor

AKADÉMIAI KIADÓ
Journals Business Centre
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E-mail: journals@akkrt.hu
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ISSN 1217-8950

AMicr 56 (2009) 1

Printed in Hungary

Acta Microbiologica et Immunologica Hungarica

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Volume 56, Number 1, March 2009

AKADÉMIAI KIADÓ
MEMBER OF WOLTERS KLUWER GROUP

Published by the financial support of
the Committee on Publishing Scientific Books and Periodicals,
Hungarian Academy of Sciences

Cover design: xfer grafikai műhely

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DOI: 10.1556/AMicr.56.2009.1.1

MICROBE-INDUCED EPIGENETIC ALTERATIONS IN HOST CELLS: THE COMING ERA OF PATHO-EPIGENETICS OF MICROBIAL INFECTIONS*

A REVIEW

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(Received: 12 January 2009; accepted: 16 January 2009)

It is well documented that the double-stranded DNA (dsDNA) genomes of certain viruses and the proviral genomes of retroviruses are regularly targeted by epigenetic regulatory mechanisms (DNA methylation, histone modifications, binding of regulatory proteins) in infected cells. In parallel, proteins encoded by viral genomes may affect the activity of a set of cellular promoters by interacting with the very same epigenetic regulatory machinery. This may result in epigenetic dysregulation and subsequent cellular dysfunctions that may manifest in or contribute to the development of pathological changes (e.g. initiation and progression of malignant neoplasms; immunodeficiency). Bacteria infecting mammals may cause diseases in a similar manner, by causing hypermethylation of key cellular promoters at CpG dinucleotides (promoter silencing, e.g. by *Campylobacter rectus* in the placenta or by *Helicobacter pylori* in gastric mucosa). I suggest that in addition to viruses and bacteria, other microparasites (protozoa) as well as macroparasites (helminths, arthropods, fungi) may induce pathological changes by epigenetic reprogramming of host cells they are interacting with. Elucidation of the epigenetic consequences of microbe-host interactions (the emerging new field of patho-epigenetics) may have important therapeutic implications because epigenetic processes can be reverted and elimination of microbes inducing patho-epigenetic changes may prevent disease development.

Keywords: carcinogenesis, DNA methylation, patho-epigenetics, epigenetic reprogramming, microbe-induced epigenetic dysregulation

* This paper was written to commemorate the 60th anniversary of the foundation of the Institute of Medical Microbiology, Semmelweis University, Budapest, Hungary.

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1. Introduction

Modified bases in the DNA of prokaryotes not only serve as *identification marks* that permit discrimination between self and foreign (invading) DNA [reviewed in 1–3], but also contribute to other vital functions, such as postreplicative mismatch correction (repair) [4], or – in *Escherichia coli* – membrane binding and segregation of the chromosome [5]. Well-characterized endodeoxyribonuclease (restriction endonuclease) and DNA methyltransferase (modification methylase) activities, recognizing the same DNA sequence, enable bacterial cells to resist infections by phage or plasmid DNA molecules that are unmethylated or methylated at different recognition sites than the host DNA. Many phages (and conjugative plasmids) can resist, however, restriction systems by either glucosylating their own genome or coding for proteins that block the activity of restriction enzymes [reviewed in 2]. A third means of escape from restriction is specifying, in bacteriophages, a methyltransferase with the same specificity as that of the bacterial host [6–9]. *Sequence-specific methylation* not only inhibits the activity of restriction endonucleases, preserving thereby the integrity of bacterial genomes, but may modulate (repress or activate) the expression of certain bacterial gene sets as well, influencing thereby bacterial virulence [10, 11].

In eukaryotes and especially in vertebrates 5-methylcytosine is the predominant modified base found in DNA [12]. In mammals, cytosine methylation is essentially confined to the sequence 5'-CpG-3' (shortly: CpG) [13]. It appears that DNA methyltransferase I (DNMT1), one of the mammalian DNA (cytosine-5)-methyltransferases, arose by fusion of a prokaryotic modification methyltransferase gene (coding for the C-terminal catalytic domain) and a second (unknown) gene, coding for a regulatory (N-terminal) domain [14–16]. A key feature of DNMT1 is its preference for hemimethylated DNA (produced during DNA replication if the parental strands were originally methylated) as a substrate. This ensures the clonal propagation of methylation patterns (*maintenance methylation*). Other mammalian methyltransferases (like the human DNMT3A and DNMT3B) play a dominant role in the methylation of unmethylated DNA substrates (*de novo* methylation) [16]. Their N-terminal domains differ from each other and from that of DNMT1 [17]. Bestor proposed that in large-genome eukaryotes DNA methyltransferases perform a new function, the *compartmentalization of the genome* [18]. This would provide an easy access to the unmethylated fraction of the genome by diffusible regulatory factors, while the usually larger, methylated fraction would remain inaccessible for them. This novel function of DNA methyltransferases is reflected in the *discontinuous and changing methylation patterns* of

vertebrate genomes: whereas prokaryotic methyltransferases modify essentially all of their recognition sites, in vertebrates there are alternating domains of methylated and unmethylated regions, and the methylation patterns of a certain region may change during development and differentiation (tissue-specific methylation patterns). The unmethylated domains, called CpG islands, are regularly found 5' to coding sequences of constitutively expressed "housekeeping" genes [19, 20]. Methylated CpG dinucleotides regularly invite methyl-CpG binding proteins that associate with histone deacetylases and histone methylases. Removal of the acetyl moieties from histone tails and methylation of selected lysine and arginine groups results in *a repressive chromatin structure silencing promoter activity* [21–23]. These alterations may switch off active promoters and stabilize the silent status of already inactivated genes. The latter effect of DNA methylation, as proposed by Riggs, may provide a *memory function*, by helping progeny cells to "remember" their proper cellular identity [24].

It is interesting to note that *an alternative system of epigenetic memory,* the Polycomb-trithorax group of protein complexes, that may operate both independently from and in concert with DNA methylation, ensures a heritable regulation of gene expression via modification of histone tails, too [25–27]. *Active promoters* are usually unmethylated at CpG dinucleotides and associate with acetylated histones H3 and H4, and histone H3 di- or trimethylated on lysine 4 (in contrast with methylation at other positions, this is a marker of actively transcribed genes).

2. Epigenetic modifications in bacteria interacting with each other

It is well appreciated that the genes encoding restriction-modification systems have undergone extensive horizontal transfer between bacterial genomes, affecting both the structure of the host genomes (by causing rearrangements similarly to mobile elements) and the epigenetic identification marks of the bacterial genomes surviving such a disturbance [28].

3. Epigenetic modifications induced by bacterial infections in multicellular eukaryotes

The epigenetic modifications induced by infecting bacteria in multicellular eukaryotes seem to be mediated by mechanisms unrelated to the transfer of bacterial restriction-modification systems, and represent *a novel research field* to be ex-

plored. Babetsis et al. described that *Campylobacter rectus*, involved in periodontal infections and associated with an increased risk for pre-term births due to placental and fetal infection in humans, could induce, in infected mice, hypermethylation in the promoter region P0 of the *Igf2* (insulin-like growth factor 2) gene in the placenta [29]. This resulted in a downregulation of Igf2 transcripts. Because deletion of the murine *Igf2* promoter region P0 blocks placental IGF2 expression and leads to reduced placental growth, followed by fetal growth restriction [30], Babetsis et al. concluded that the intra-uterine growth restriction they observed in *Campylobacter rectus* infected animals was a consequence of the epigenetic alteration induced by the bacterial infection in the murine placenta (Table I).

Helicobacter pylori is phylogenetically closely related to *Campylobacter rectus* and shares a common antigen (GroEL) with the latter [31]. *Helicobacter pylori* infection, the most important acquired risk factor for gastric cancer in humans [32], has been shown to induce aberrant DNA methylation in the gastric mucosa of healthy volunteers [33]. One of the genes inactivated due to *Helicobacter pylori* infection-induced promoter methylation encodes E-cadherin, *an adhesion molecule involved in tumor invasion and metastasis*. The inactivation of the E-cadherin promoter was considered to be an early event in gastric carcinogenesis [34], and could be achieved in human gastric cell lines cocultivated with *Helicobacter pylori* or treated with interleukin-1β *in vitro* [35]. Similarly, persistent *Helicobacter pylori* infection in the middle/lower stomach may induce inactivation (by promoter hypermethylation) of *RUNX3, a possible tumor suppressor gene for gastric cancer* [36]. Hypermethylation of a set of distinct promoters (located within CpG islands) occurs frequently in gastric cancer [37], and in gastric mucosa one could distinguish between cancer-associated and *Helicobacter pylori*-associated hypermethylation [38]. The latter may be determined by Polycomb repressive marks (see Introduction) localized to the hypermethylated genes of stem or progenitor cells in gastric mucosa [38]. Ushijima suggested that *Helicobacter pylori* infection induces both temporary and permanent methylation, in progenitor or differentiated cells and stem cells, respectively; methylation in the *stem cell fraction* is expected to be proportional to gastric cancer risk [39]. In patients with enlarged fold gastritis, some of the alterations in the methylation patterns proved to be reversible after eradication of *Helicobacter pylori* [40] (Table I).

Table I

Microbe-induced epigenetic dysregulations
(For detailed explanation, see the text)

Microbe/effector protein	Mechanism of action	Consequence
Bacteria		
Campylobacter rectus	?	Silencing of *Igf2* P0 promoter by CpG methylation in the placenta
Helicobacter pylori	Polycomb-repressive marks pinpoint the promoters to be silenced	Silencing of selected promoters by CpG methylation
Viruses		
Epstein-Barr virus, LMP1	Up-regulation of DNMT1, 3A, 3B via the JNK-AP-1 pathway	Silencing of E-cadherin promoter
KSHV, LANA	Association with DNMT1, 3A, 3B, relocalization of DNMT3A and DNMT3B; recruiting DNMTs to cellular promoters	Silencing of H-cadherin promoter
Human adenovirus, E1A	Stimulation of E2F activity, up-regulation of DNMT1; association with DNMT1, stimulation of DNMT1 activity	?
BK virus, large T	Stimulation of E2F activity, up-regulation of DNMT1	?
Human papillomavirus, E7	Association with DNMT1, stimulation of DNMT1 activity; increasing histone acetylation	? ? Activation of *E2F* and *CDC25A* promoters
SV40, large T	Stabilization of DNMT levels;	*De novo* methylation of selected cellular genes by DNMT3B (?)
Hepatitis B virus, pX (HBx)	Up-regulation of DNMT1 via the cyclin D1-CDK4/6-pRb-E2F1 and p38MAPK pathways; up-regulation of DNMT3A1 and DNMT3A2; Down-regulation of DNMT3B	Silencing of tumor suppressor genes Hypomethylation of satellite 2 repeats
HIV, early expressed proteins	Up-regulation of DNMT1 via the AP1 pathway	Silencing of *IFN-γ* and *GNE* promoter
Hepatitis C virus, core protein	Up-regulation of DNMT1 and 3B	Silencing of IFN-regulated genes (?)

4. Restriction-modification systems of viruses infecting unicellular eukaryotes

Rapid changes in marine algal populations were associated with viral infections [41]. Chlorella viruses are double-stranded-DNA-containing viruses replicating in certain strains of the unicellular green alga Chlorella. They encode multiple DNA methyltransferases and site-specific endonucleases. The viral endonucleases degrade the (unmodified?) host cell DNA whereas at least one of the viral DNA methyltransferases was suggested to protect the viral DNA from degradation in the infected cell [42–44].

5. DNA (cytosine-5-)-methyltransferases of viruses infecting multicellular eukaryotes

The genomes of two frog herpesviruses, Ranid herpesvirus 1 (the etiological agent of the Lucké renal adenocarcinoma of the North American leopard frog, *Rana pipiens*), and Ranid herpesvirus 2, encode a putative DNA (cytosine-5-)-methyltransferase and their virion DNA is extensively methylated at CpG dinucleotides [45]. It is not clear (although most probable) at present, whether the putative viral enzyme is responsible for the modification of the viral genome. It also remains to be established, whether it affects the host cell genome.

The genomes of certain iridoviruses that infect vertebrate hosts (lymphocystis disease virus, infecting different fish species, and frog virus 3) also encode DNA (cytosine-5-)-methyltransferases [46, 47]. Frog virus 3 DNA is newly synthetized and *de novo* methylated in the nuclei of *in vitro* infected cells. This process is followed by the export of the viral DNA into the cytoplasm for viral assembly [48]. The high degree of methylation of the frog virus 3 genome suggested that cells infected with frog virus 3 are capable to transcribe even highly methylated promoters. This phenomenon is due to the action of a virus-induced trans-acting protein [49, 50].

6. Induction of epigenetic dysregulation in virus-infected vertebrate cells

Cancer formation involves genetic and epigenetic alterations resulting to the loss of multiple growth-control mechanisms and changes of the social behaviour of the affected cells [51, 52]. Oncoviruses provided early cues as to the *ge-*

netic background of neoplastic development, and recently their interaction with *epigenetic* regulatory mechanisms became apparent as well (reviewed in [53]). In addition, certain viruses not known to be capable of immortalization or malignant transformation of cells were also found to induce epigenetic dysregulation in infected host cells.

6.1. The role of virus-induced epigenetic alterations in malignant transformation

A series of viruses encoding oncoproteins (and sometimes also non-translated RNAs involved in oncogenic transformation) have well-documented links to human cancer [54]. Recent studies demonstrated that viral oncoproteins, in addition to their well-characterized interactions with cellular signaling pathways, may affect the epigenetic regulatory systems of infected cells and alter (usually silence) the expression of cellular genes and gene sets (Table I).

6.1.1. Epigenetic alterations in Epstein-Barr virus associated neoplasms

Epstein-Barr virus (EBV), a human herpesvirus, is associated with a wide variety of neoplasms (reviewed in [55]). The expression of latent, growth-transformation-associated viral genes is under a strict, host-cell-phenotype dependent epigenetic control. Latent EBV infection apparently results in hypermethylation of a set of cellular promoters in Burkitt's lymphoma [56], nasopharyngeal carcinoma [57], Hodgkin's disease [58, 59], and EBV-associated gastric carcinoma [60, 61], but the exact mechanism(s) mediating epigenetic dysregulation are unknown in most cases. Tsai et al. observed, however, that expression of latent membrane protein 1 (LMP1), an EBV encoded oncoprotein, up-regulated the expression and activity of cellular DNA methyltransferases 1, 3a and 3b *in vitro* [62]. This resulted in hypermethylation of the E-cadherin promoter and downregulation of E-cadherin gene expression, similarly to the phenomenon observed in *Helicobacter pylori* infected gastric mucosa (see above, 3; see also in [33]). In a follow-up study it was demonstrated that LMP1 activates *DNMT1* via the c-jun NH(2)-terminal kinase/activator protein-1 (JNK-AP-1) signaling pathway [63]. In addition, formation of a transcriptional repression complex (composed of DNMT1 and histone deacetylase) could be detected on the E-cadherin promoter as a consequence of LMP1 action. How this repressor complex was targeted to the E-cadherin promoter, however, remains to be elucidated.

6.1.2. Epigenetic alterations in cells carrying latent Kaposi's sarcoma-associated herpesvirus genomes

Kaposi's sarcoma associated herpesvirus (KSHV; so-called human herpesvirus 8, HHV-8) is the causative agent of Kaposi's sarcoma, primary effusion lymphoma and multicentric Castelman's disease [reviewed in 64]. The latency associated nuclear antigen (LANA) encoded by KSHV was reported to be associated with DNMT1, DNMT3A and DNMT3B [65]. In addition, LANA relocalized DNMT3A (and to some extent also DNMT3B) from the nuclear matrix to the chromatin. Because LANA preferentially interacted with repressed cellular promoters in endothelial cells and promoted *de novo* methylation of the H-cadherin *(CDH-13)* promoter, Shamay et al. suggested that LANA induces promoter-specific *de novo* DNA methylation by recruiting a *de novo* DNA methyltransferase activity [65]. Similarly, LANA silenced the promoter of transforming growth factor-β type II receptor (TβRII) in primary effusion lymphoma cells by *site-specific induction of CpG methylation and deacetylation of proximal histones* [66].

6.1.3. Interactions of adenovirus, papillomavirus, polyomavirus, and hepatitis B virus encoded oncoproteins with epigenetic regulatory mechanisms

Oncoproteins of adenoviruses and papovaviruses activate the DNA replication machinery of the host cell via inactivating members of the retinoblastoma pocket protein family and releasing thereby "activating" E2F transcription factors that control the coordinated transcription of genes involved in DNA replication and cell cycle progression [67, 68]. *DNMT1* was found to be an E2F target as well [68]. Accordingly, the oncoproteins E1A and BK virus (BKV) large T, encoded by a human adenovirus and a human polyomavirus, respectively, could stimulate E2F activity and activate thereby the transcription of the *DNMT1* gene [70]. It is interesting to note that E1A not only affects transcription of *DNMT1*, but similarly to the E7 oncoprotein of human papillomavirus (HPV) type 16, E1A can also *directly associate* with DNMT1 and stimulate its methyltransferase activity *in vitro* [71]. HPV type 16 E7 has an additional action as well, because it binds both the retinoblastoma protein (pRb) and histone deacetylase, *and increases histone acetylation* at the activated *E2F1* and *CDC25A* promoters in human foreskin keratinocytes [72].

Similarly to the human polyomavirus BKV, the related simian virus 40 (SV40) also affects epigenetic regulation. SV40-induced immortalization of fibroblasts resulted in a stabilization of DNA methyltransferase levels [73], and

expression of DNA methyltransferase 3b was found to be essential to oncogenic transformation induced by SV40 large T antigen in a lung cancer model [74]. *De novo* methylation of selected cellular genes was associated both with immortalization and oncogenic transformation in these experiments. Several reports described detection of SV40 DNA sequences and concomitant alterations in the methylation profiles of selected cellular genes in human neoplasms [75–77]. The exact mechanism of aberrant methylation in these cases remains to be determined.

The hepatitis B virus encoded X protein (HBx or pX) is implicated in the pathogenesis of hepatocellular carcinoma. As a pleiotropic regulator, pX affects multiple key signaling pathways [78–80]. Although the interaction of pX with pRb was less intensively studied than that of the oncoproteins encoded by adeno- and papovaviruses, it turned to be that pX could also overcome the inhibition of E2F1 activity by pRb [81]. In addition, pX up-regulated cyclin D1 and activated DNMT1 expression via the cyclin D1-CDK4/6-pRb-E2F1 pathway, that resulted in the DNA-methylation-mediated decrease in the level of $p16^{INK4a}$ (a tumor suppressor protein) in HepG2 cells [82]. In hepatocytes it was demonstrated that pX induces E2F1 release via the p38MAPK pathway as well [83]. In addition to DNMT1, two variants of DNMT3A (termed DNMT3A1 and DNMT3A2, translated from differentially spliced trancripts of the *DNMT3A* gene) were also found in increased amounts in liver cells transfected with a pX expressing vector [84]. This resulted in *regional hypermethylation* of specific tumor suppressor genes. It is interesting to note, however, that pX *down-regulated* DNMT3B in the very same cells. Because DNMT3B is involved in the methylation of satellite 2 repeat sequences, pX induced a *global hypomethylation* of these repeats [84]. As far as I know, this is the first observation connecting a viral oncoprotein with global DNA hypomethylation, a phenomenon as widespread in neoplastic cells [85] as regional hypermethylation [52]. A CpG rich sequence *hypomethylated* in hepatocellular carcinomas carrying integrated HBV genomes was found in the peri-centromeric region of human acrocentric chromosomes in an independent study [86]. This sequence, despite its similar localization, differed from the typical alpha satellite sequence. Regarding the regions hypermethylated in HBV positive hepatocellular carcinomas, Shim et al. found that hypermethylation of the *P16* gene starts at an early stage of hepatocarcinogenesis: cirrhotic nodules (regarded as preneoplastic lesions) and dysplastic nodules, surrounding HBV positive hepatocellular carcinoma lesions in tissue sections, showed *P16* hypermethylation in approximately 62% and 70% of cases, respectively [87]. Hypermethylation of E-cadherin promoter was repeatedly observed and attributed to pX [88–90]. It is noteworthy, however, that in contrast the carcinomas of other organs, hepatocellular carcino-

mas expressing E-cadherin frequently invade blood vessels [88]. The tumor suppressor gene *RASSF1A* (Ras association domain family 1A) and the *GSTP1* gene (coding for the π-class glutathione S-transferase, an enzime involved in protecting against electrophilic carcinogens) is also frequently inactivated by CpG island methylation in HBV positive hepatocellular carcinomas [91, 92].

6.2. Cellular dysfunctions induced by viruses via epigenetic reprogramming of the host cells

Infection of T-helper cells with human immunodeficiency virus (HIV) results in *dysregulation of cytokine gene expression*. Mikovits et al. observed that downregulation of interferon gamma (IFN-γ) expression after acute HIV infection was due to increased DNA methyltransferase expression resulting in an increased cellular DNA methylation in general, and methylation of the *IFN-γ* promoter [93]. As far as I know, this was *the first report on an epigenetic change induced by a microbe pathogenic to humans*. In a follow-up study it was demonstrated that HIV selectively up-regulated DNMT1 (but not DNMT3A and DNMT3B) expression and silenced the *P16* promoter [94]. Integration of the proviral HIV genome was not necessary to induce epigenetic dysregulation in the virus infected cells.

In addition to the dysregulation of cytokine gene expression, HIV infection can also affect the sialylation status of glycoproteins involved in lymphocyte homing, recirculation and survival, because the promoter of the *GNE* gene encoding UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase is also switched off by hypermethylation in HIV infected T cells, resulting in a decrease of CMP-sialic acid synthesis and *hyposialylation* of proteins [95] (Table I).

Up-regulation of DNMT1 expression was found to be a function of the early expressed HIV proteins affecting the AP1 pathway [96]. The regulatory region (long terminal repeats, LTRs) of the proviral HIV genome was also demonstrated to be regulated by DNA methylation, although its silencing was imperfect in certain clonal cell lines [97, 98].

Hepatitis C virus (HCV) infection is an important cause of chronic liver disease in humans. Standard therapy using pegylated interferon alpha (IFN-α) may fail due to viral interference with IFN-α signal tranduction through the Jak-STAT pathway, disruption of type I IFN receptors, or *epigenetic silencing of IFN-stimulated genes in HCV replicon harbouring cells* [99, 100]. The latter may be due to the activation of DNA methyltransferase 1 and 3B by the *core protein* of HCV, a process that results in silencing, via hypermethylation, of the E-cadherin promoter

[101]. The E-cadherin promoter, as well as the promoters of a series of tumor suppressor genes were found to be inactivated in HCV-associated hepatocellular carcinoma, similarly to the HBV-associated neoplasms (see above, 6.1.3) [102–104] (Table I).

7. Macroparasite-induced DNA hypermethylation in host cells

Infection of the alfalfa plant by the parasite plant *Cuscuta reflexa* increases the amount of 5-methylcytosine in stem DNA of the host species [105]. This suggests that macroparasites may also elicit epigenetic alterations in host cells. Thus, one may speculate that similarly to macroparasites infecting plants, macroparasites infecting vertebrates (helminths and arthropods [106]; also fungi) may induce epigenetic reprogramming of the host cells interacting with them as well, like certain microparasites (bacteria and viruses) do (see above, 3; 6). Protozoa, also considered to be microparasites [107], may elicit epigenetic dysregulation in the cells of their multicellular hosts in a similar manner.

8. Future directions: Patho-epigenetics of microbial infections

Honess et al. noticed that the genome of Epstein-Barr virus, a human gammaherpesvirus establishing latency in memory B cells (reviewed in [108]), is deficient in CpG dinucleotides, similarly to higher eukaryotic DNA sequences that are methylated at certain cytosine residues [109]. On this basis they suggested that the latent genomes of gammaherpesviruses are subjected to CpG methylation in their host cells. This turned to be the case (reviewed in [110]). Recent data suggest, however, that the double-stranded DNA (dsDNA) genomes of other herpesviruses (members of *Alpha-* and *Betaherpesvirinae;* most of these genomes are not deficient in CpG, see [111]) are invariably unmethylated (both in the virions and in latently or productively infected cells), and their latent genomes are targeted, accordingly, by cellular epigenetic regulatory mechanisms other than CpG methylation (see [112] for review). In contrast, the genomes of other dsDNA viruses and the proviral genome of retroviruses (like HIV) are similar to gammaherpesvirus genomes: they are frequently subjected to CpG methylation (reviewed in [113]; see also above, 6.2). Thus, the idea that the expression of latent viral dsDNA genomes is controlled by epigenetic regulatory mechanisms seems to be well documented by now.

Whereas certain dsDNA viral genomes are targeted by epigenetic regulatory mechanisms in the infected host cells, proteins encoded by viral genomes (as demonstrated above, see 6.1, 6.2) may affect, in parallel, the activity of a set of cellular promoters by interacting with the very same epigenetic regulatory machinery. This may result in transient or permanent *epigenetic dysregulation* and subsequent cellular dysfunctions that may manifest in pathological changes (disease). I propopse that other microparasites (bacteria, see 3; protozoa) and also macroparasites (helminths, arthropods, fungi) may induce pathologic changes in a similar manner, i.e. by induction of *epigenetic reprogramming* of certain host cells. The genetic background of a series of human diseases is well elucidated by now (pathogenetics), and the epigenetic (or combined genetic and epigenetic) background of certain human diseases was also clarified (summarized in [16]). I got the impression that the elucidation of pathological changes elicited by epigenetic dysregulation is gaining momentum: a new field, *patho-epigenetics* was born. As to microbial infections, this new field of patho-epigenetics is most important and promising, because epigenetic processes can be reverted and their reversion may have therapeutic consequences. Also, elimination of microbes inducing patho-epigenetic changes may efficiently prevent disease development (see [40, 114, 115]). Although in this stage it looks like that first of all *chronic, persistant, or latent infections* induce epigenetic reprogramming of the host cells, epigenetic dysregulation may become more and more important in *acute infections*, too (see 3, 6.2).

Acknowledgement

I thank Kálmán Szenthe for critical reading of the manuscript.

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 21–51 (2009)
DOI: 10.1556/AMicr.56.2009.1.2

CLINICAL MICROBIOLOGY OF EARLY-ONSET AND LATE-ONSET NEONATAL SEPSIS, PARTICULARLY AMONG PRETERM BABIES*

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(Received: 16 January 2009; accepted: 19 January 2009)

Prematurity has got special challenge for clinicians and also other medical staff, such as microbiologists. Immature host defense mechanisms support early-onset sepsis, which can be very serious with very high mortality. While the past decade has been marked by a significant decline in early-onset group B streptococcal (GBS) sepsis in both term and preterm neonates, the overall incidence of early-onset sepsis has not decreased in many centers, and several studies have found an increase in sepsis due to gram-negative organisms. With increasing survival of these more fastidious preterm infants, late-onset sepsis or specially nosocomial bloodstream infection (BSI) will continue to be a challenging complication that affects other morbidities, length of hospitalization, cost of care, and mortality rates. Especially the very low birthweight (VLBW) infants sensitive to serious systemic infection during their initial hospital stay. Sepsis caused by multiresistant organisms and *Candida* spp. are also increasing in incidence, has become the most common cause of death among preterm infants. This review focuses on the clinical microbiology of neonatal sepsis, particularly among preterm babies, summarizing the most frequent bacterial and fungal organisms causing perinatally acquired and also nosocomial sepsis.

Keywords: neonatal sepsis, preterm newborn, early-onset sepsis, late-onset sepsis, nosocomial infection

* This paper was written to commemorate the 60th anniversary of the foundation of the Institute of Medical Microbiology, Semmelweis University, Budapest, Hungary

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Introduction

Even though recent marked advances in intensive care skill and facilities have improved the survival rate of high risk neonates, they have also increase the frequency of invasive procedures. Neonates admitted to neonatal intensive care units (NICUs) are at a greater risk of developing neonatal sepsis. The reported incidence of neonatal infection in NICUs ranges from 1 to 8.6 per 1,000 live births, although underreporting is common. Neonates who develop sepsis also have significantly prolonged hospital stay increasing the cost of neonatal care. Culture-proven sepsis may occur in up to 20% of NICU admission.

Multicenter and institutional surveillance data indicated that BSI is the most common nosocomial infection in NICUs. The variability of endemic nosocomial infection rates among centers with similar patient populations suggests that the rigorous implementation of health care practice and the existence of predisposing factors will influence the nosocomial infection rates.

Although morbidity and mortality remain high for both early-onset and late-onset sepsis despite the use of potent antimicrobials, there is also some evidence that there has been a change in the epidemiology of sepsis, with gram-negative organisms once again being predominant. Therefore, it is important for neonatal units to maintain ongoing surveillance and reflect on their experience. Special question is the relevance of surveillance cultures. The results of studies might support a satisfactory predictive value negative for surveillance cultures.

Definitions

Neonatal sepsis

Infections in newborns often present with sepsis, with or without focal signs of infection. Neonatal sepsis generally refers to systemic symptomatic bacterial, fungal and viral infections that may be associated with any gradation of symptomes, from only subble feeding to frank septic shock. Often the early signs of neonatal sepsis are nonspecific, such as temperature instability, lethargy, poor feeding, apnea, bradycardia, respiratory distress, vomiting, diarrhea, abdominal distension, jitteriness and seizures, or just an unexplained hyperbilirubinaemia. In addition, as diagnostic procedures are different in the Neonatal Intensive Care Units (NICUs), the definitions of such infections for children aged < 1 year of the Centers of Disease Control and Prevention (CDC) are less suitable in this patient

population [34]. CDC definitions for (premature) neonates are still lacking. The lack of standardized definitions for nosocomial infection in the NICU and the huge diversity in patient mix in different NICUs makes comparison of studies on the incidence of infection in neonates difficult [27, 85]. Some authors tried to find more useful scoring process to predict which infants have nosocomial sepsis. For example the NOSEP scoring system assigns a point score for results of special laboratory investigation (CRP, neutrophil fraction, platelet count) and the length of parenteral nutrition and fever [53, 95]. Other subcohort investigation using the Clinical Risk Index for Babies (CRIB) showed that the CRIB was not predictive for infection, but with the CDC criteria, only 75% of BSI would have been identified [89, 93].

In summary, in mainly every diagnostical guideline contained, that the diagnosis of culture-proven sepsis required the recovery of a recognized pathogen from culture of the blood or recovery of a commensal organism from at least two blood cultures [53, 95] beside the other laboratory test or clinical sign.

Prematurity

Preterm delivery is defined as delivery at less than 37 weeks of estimated gestation. It gives some special risk factors to infection because of the weaker immune function, as well as in complement and phagocytic activity; lack of the IgG antibody from the mother and specially lack of antibodies to major virulence antigene, the type III capsular carbohydrate of *Streptococcus agalactiae*. They have got poor skin and mucosal barriers, since the skin may be parcment-like with little subcutaneous tissue.

Birth weight, low birthweight

Since it is well known that the incidence of neonatal sepsis is inversely proportional to birth weight and gestational age, only recent studies covering the topic of neonatal sepsis have addressed very low birthweight (VLBW) and extremely low birthweight (ELBW) infants separately from other preterm and term infants [41].

Neonates could be stratified into four birthweight categories: extremely low birthweight 500–999 g (ELBW), very low birthweight (VLBW) 1000–1499 g, low birthweight (LBW) 1500–2499 g and normal birthweight > 2500 g [27].

Onset/Time

Neonatal infections are usually classified according to time and mode of onset.

Early-onset sepsis (EOS)

A variety of different classifications concerning time at onset of symptoms has been used in the literature so far, ranging from 24 hours to 7 days for "early-onset" sepsis. In many reports the sepsis has been classified as early-onset sepsis (EOS), if the infection starts before 72 hours of life [11, 54, 84, 90]. In many other reports according to Vesikari et al. [9, 31, 38, 94] the infections with "early-onset" defined as up to the age of 1 week, may also originate from intrauterin colonization, but may also be aquired during delivery by contact with pathogens in the birth canal originating from the NICU environment. In addition, they use "very-early-onset" disease, if it starts <24 hours of life, when infection probably occurred in utero, justifying the classification of this group as a single entity.

The exact time period defined as early-onset infection is not very important, as 80–90% of infections in the first week of life have their onset in the first 2 days of life [11].

Late-onset sepsis (LOS)

The late-onset sepsis (LOS) defined if it occurred after 3 days or after the first week of life [9, 31, 54, 90]. It is probably the result of nosocomial infection [31, 57]. Some reports use special additional definitions, such as late-late-onset sepsis (LLOS) before 2 months or very-late-onset neonatal sepsis (VLONS), defined as sepsis starting 60 days after birth [11, 63]. These definitions have contributed greatly to diagnosis and treatment by identifying which microorganisms are likely to be responsible for sepsis during these periods and the expected outcomes of infection. The mean age at the time of diagnosis of late-onset sepsis ranged 14 to 36 days [9, 11, 31, 37, 85].

Risk factors

Risk factors for EOS

Several risk factors for early-onset postnatal infections have a very strong influence on infection rates, which are especially *maternal factors*. Prolonged premature rupture of membranes, septic or traumatic delivery, maternal carriage of *Streptococcus agalactiae* (GBS), maternal poverty, pre-eclampsia, cardiac disease, diabetes mellitus are significant risk factors [31, 84]. Studies indicate that intra-amniotic infection complicates up to 10% of all pregnancies and up to 2% of labors at term. While maternal mortality caused by chorioamnionitis is rare, neonatal mortality directly related to this ranges from 1% to 4% for infants in term and more than 10% for preterm infants [22]. Naturally, the *neonatal factors* are very strong connection with maternal factors. The rate of infection is 20–25% for premature infants born to mothers with amnionitis, while in case of healthy full-term infants born to mothers with amnionitis is approximately 1% [11].

The *neonatal factors*, especially prematurity, lower gestational age at birth, fetal anoxia [9, 11, 84], have got strong connection with EOS, while low APGAR score at 5 minutes does not have [31]. Generally, the male gender is significant factor to EOS [11, 31] with some exception [84]. There were no significant differences in the rates of EOS between infants whose mothers received antibiotics during their hospitalization or 72 hours before delivery and those whose mothers did not receive antibiotics [84]. Sometimes the faulty examining technique may introduce organisms during an obstetric examination or when monitoring equipment is used (scalp electrodes) [77].

Risk factors for LOS

The risk factors for late-onset infections cannot be separated from those for early-onset infections because the most common reason for prolonged neonatal hospitalization, long stay in nursery is the prematurity.

Generally, in every study, the birth weight (BW) and gestational age (GA) were the most important risk factors with the strongest statistical relationship to LOS. Since only 7–10% of infants with BW between 1, 000 and 1, 500 g develop sepsis compared with 15 35% of infants with BW of 1, 000 g and 28-50% of infants with BW of 750 g [6, 17, 38, 55, 82, 85, 90].

The infection rate is inversely related to GA. Almost 46–54% of the neonates born at <25 weeks' gestation developed late-onset sepsis. This rate of infection declined to 29% at 25 to 28 weeks, 10% at 29 to 32 weeks [11, 38, 54, 83, 85].

Risk factors for nosocomial sepsis could be differenciated as intrinsic or extrinsic. Intrinsic risk factors include the relative immunodeficiency of the neonate, and compromised portals of entry for potential pathogens including the immature barrier function of the skin and the gastrointestinal tract. The immature and fragile skin of ELBW infants is a poor epidermal barrier to prevent invasion by colonizing bacteria. Infants most often develop nosocomial sepsis caused by endogenous flora and are themselves implicated as the major reservoir of potential pathogens that can be transmitted from patient to patient, often via the hands of staff. Use of antimicrobial agents, delayed enteral feeding, and prolonged hospitalization are factors that can lead to altered endogenous flora among infants in the NICU, when compared with healthy, full-term infants [11, 83, 93]. Gram-negative enteric bacteria are usually derived from the patient's endogenous flora, which may have been altered by antecedent antibiotic therapy or populated by resistant organisms transferred from the hands of personnel (the major means of spread) or contaminated equipment. Therefore, situations increasing exposure to these bacteria, hygienic problem (e.g. crowding, nurse: patient ratios > 1:1, inadequate hand washing) result in higher rates of hospital-aquired infection [98]. One study found that nosocomial infection rate was most strongly correlated with patient density [2], while others correlated staphylococcal or *E. cloaceae* outbreaks with overcrowding [1, 18, 32].

Extrinsic risk factors include medical treatments, devices and invasive procedures. Use of umbilical and other central line was associated with a significantly increased rate of infection, while the rate of infection increased with an increasing duration of central venous catheter use [11, 20, 27, 31, 38, 57, 83]. More than a decade ago, the CDC recommended that NICU rates be expressed as device-associated, device-days rates, and be adjusted by BW i.e., <1000 g, 1001 1500 g, 1501–2500 g and >2500 g [17].

Endotracheal intubation, assisted ventilation and surgery (presence of surgical wounds and drains) are also important risk factors to LOS [11, 31, 38, 83, 91].

Epidemiology

Rate

The rate of all neonatal sepsis has been 0.5 8 per 1000 live births since 1980 in the world, with higher rates in developing countries. The rate of sepsis in infants born at any hospital varies according to the economic standards, availability of prenatal care, and variations in perinatal risk factors [29].

The incidence of EOS in centers ranged from 4 to 27 per 1000 live births [54, 84].

The incidence of nosocomial infections in NICUs is high; it has been reported to occur in up to 39% of all neonates [9, 17, 25, 31, 32, 42, 58, 59, 69, 72, 82, 86, 104] ranging from 5.2 to 30.4 infections per 100 patients. Despite advances in neonatal care, overall case-fatality rates from sepsis range from 2% to as high as 50% [90]. Some studies revealed slight decreasing rate of nosocomial bloodstream infections [31, 99], while others found slight increasing rate [11].

Among nosocomial infections, the bloodstream infection is the most frequent type [17, 27, 57, 69, 85] in all birth weight groups.

Number of septic episodes

The chance of having multiple episodes and the number of episodes per infant decreased with increasing BW. Multiple sepsis episodes are more common in the lowest-birthweight categories, with almost 40% of infants with birth weights of 750 g having two or more episodes [54, 83, 85, 90, 99].

Hospital stay

In studies, the average length of NICU stay for all weights was approximately 20 days, and it showed a wide variation among BW categories: 12–40 days [17]. Particularly the duration of hospitalization was significantly longer for VLBW infants who had LOS than for those without LOS. The mean length of stay: 79–86 vs 60–61 days [17, 83].

Mortality

Many reports have shown that the major impact of preterm delivery and low birthweight on mortality originates from invasive infection. The fatality rate is 2 to 4 times higher in LBW than in full-term infants [38, 54]. Early mortality was associated with a lower age at the time of onset of sepsis, lower gestational age and birth weight and it also dependent on the associated microorganism.

EOS usually progresses rapidly and has multiorgan involvement. Infants with EOS had a significantly higher risk of respiratory distress syndrome, severe intraventricular hemorrhage or periventricular leukomalacia [54, 84]. It is an important cause of neonatal death. Mortality ranges from 4 to 50% [10, 11, 29, 31, 70, 84, 99]. Particularly, mortality of the early-onset GBS sepsis is 2–30%.

Mortality of LOS is usually much lower than in EOS [9, 31]. The incidence of nosocomial sepsis in NICUs ranged from 5 to 30 per 100 patients [11]. The clinical characteristics and the early mortality rates of infants varied according to the type of pathogen responsible for the LOS [38, 90]. In most studies, *P. aeruginosa* infections had the highest mortality (22–62%) compared to infants infected by other pathogens [38, 54, 83]. Death rates were also high for infants infected with *Candida albicans* (28%) and other gram-negative pathogens (13–22%), while the death rate was lower among babies infected by *coagulase-negative staphylococci (CoNS)* (2–10%) [9, 11, 28, 54, 83, 85].

Infection patient-day rate (per 1000 patient day)

The average number of episodes per 1000 days in hospital was 2.59–13.9, respectively [38, 83, 93, 99].

Microbiological diagnosis

Early diagnosis is important and requires awareness of risk factors (particularly in LBW neonates) and a high index of suspicion when any neonate deviates from the norm in the 1st few weeks of life. Neonates with suspected sepsis, and those whose mother was thought to have chorioamnionitis, should have blood culture, urine culture, and lumbar puncture, if clinically feasible, as soon as possible. Diagnosis is confirmed by isolation of a pathogen in culture.

Major pathogens of neonatal sepsis vary with geographic area and time, and significant interinstitution variation in the incidence, especially of LOS, has also been reported. The microbial causes of late-onset neonatal sepsis have changed over time as they are heavily influenced by newer developments in the care for very low birthweight newborns.

It will be important, however, to continue surveillance of neonatal septicaemia in order to follow closely changes in trends and risk factors, to obtain information on which to base empiric antibiotic therapy and to react rapidly in case of major changes in susceptibility patterns and occurrence of outbreaks. These trends are important to recognise because they provide the basis for rational empiric antimicrobial therapy.

The microbiology laboratory must produce results that are correct and quality is a major issue. All laboratory results should be reported as quickly as possible. In most situations it is adequate to have the information on the hospital computer system and send out printed reports. The microbiological laboratory may be the first to notice an infection control problem and has been described as an 'early warning system' for infection control problems [101].

Cultures of a newborn being evaluated for sepsis should routinely include one or more blood cultures, urine culture, and cerebrospinal fluid culture [4].

Blood culture

The "gold standard" for diagnosing neonatal sepsis remains the blood culture, the isolation of an organism from a blood culture of a neonate with clinical symptoms of infection. At birth the indications for blood sampling are clinical signs of infection or maternal risk factors for congenital septicaemia (premature rupture of membranes, premature labour, and maternal fever).

In many cases, blood cultures are negative in the face of strong clinical indicators of septicemia. Given the widespread use of intrapartum antibiotics with preterm labor, the incidence of clinical sepsis (symptoms and abnormal laboratory findings with no growth from blood culture) is significantly higher than the incidence of culture-proven sepsis. False-negative blood cultures may also result from insufficient sample size. Due to technical constraints, often only a single peripheral blood culture is obtained from a septic-appearing neonate, and in most studies the isolation of an organism from one blood culture is considered evidence of sepsis.

Only one of every five evaluations for sepsis with a blood culture yielded a microorganism. This underscores the finding that 80% of the time, empiric antibiotics will be given when no organism is isolated from culture.

Umbilical vessels are frequently contaminated by organisms on the umbilical stump, especially after a number of hours, so blood cultures from umbilical lines may not be reliable.

If the same microorganism is isolated from an infant on more than one occasion within a 7-day period this is counted as one single episode of sepsis.

Cultures that are positive for organisms that are generally considered to be contaminants-including *Corynebacterium* spp., *Micrococcus* spp., *Bacillus* spp., *Propionibacterium* spp. and *diphteroids* are generally signed as contaminated specimens.

The significance of CoNS from blood cultures from febrile patients can be difficult to determine because their recovery may reflect bloodstream infection or blood culture contamination (a colonization of intravascular catheters or transient bacteremia). The differentiation is particularly difficult in pediatric patients, from whom only a single blood culture is frequently obtained. There is, no gold standard for diagnosing true bacteremia when blood cultures yield CoNS. Several laboratory criteria have been proposed as predictors, including culture 2 blood samples obtained simultaneously from different sites by means of a strictly aseptic technique or one positive culture and a blood C-reactive protein level greater than 1 mg per deciliter within two days after the blood culture for definitive infection. Possible infection is defined by one positive culture and treatment for at least five days with vancomycin, oxacillin, a semisynthetic antistaphylococcal agent, or another drug to which the organism was susceptible [84]. Some authors defined that whenever CoNS and another pathogen were identified in the same blood culture, only the other pathogen was recorded in the database [54]. Implied in the criterion that multiple, positive cultures predict bloodstream infections that multiple positive cultures predict BSI is that the same species and clone of CoNS grew in these cultures. PFGE to perform strain typing and reference methods to obtain species-level identification are very useful to understand better and possible to improve the interpretation of CoNS blood-culture isolates.

Approximately half of clinicians routinely interpreted isolation of CoNS from a single blood culture as indicative of sepsis and completed a course of antimicrobial therapy.

Furthermore, with modern blood culturing techniques, including the common use of automated, continuous monitoring blood culture systems, the clinician

is tipically notified of a positive blood culture within 24 hours of incubation and clinically significant isolates are almost always noted within 48 hours.

If catheter-associated sepsis is suspected, a culture should be obtained through the catheter as well as peripherally. Catheter-related sepsis is defined as both a positive blood-culture and a culture-positive intravascular catheter tip with the same microorganism, including an identical resistance pattern, in a patient with clinically suspected sepsis and no other source of infection [8, 9, 17, 26, 36, 70].

Joint fluid, peritoneal fluid

They are not performed routinely, but should be performed if there is evidence of focal infection.

Focal infections of intestinal tract are also common in preterm infants and may occur with or without a positive blood culture.

Liquor cerebrospinal fluid (LCF)

Focal infections of central nervous system (CNS) are also common in preterm infants and may occur with or without a positive blood culture. LCF should be performed in a neonate with suspected sepsis as soon as he is able to tolerate the procedure. Because GBS pneumonia manifesting in the 1st day of life can be confused with hyaline membrane disease, LCF is often performed routinely in neonates suspected of having these diseases.

Superficial cultures

The use of superficial cultures, such as cultures of skin, umbilicus and gastric contents, may be helpful in determining of organisms have been transferred from mother to newborn, if obtained immediately after birth.

Urine

Urine cultures are useful when a blood culture grows a pathogen and the specific focus of infection needs to be classified. Bagged urine from a neonate is

frequently contaminated with skin flora. Urine cultures should be obtained by urethral catheterization or suprapubic bladder aspiration. Absence of pyuria does not rule out urinary tract infection.

Surveillance cultures

CoNS, particularly *Staphylococcus epidermidis*, are the most common organisms found in mucocutaneous sites and the nasopharynx during the first week of life. The prevalence of these organisms increases from 9% at birth to 78% during the second week of life. Gastrointestinal tract colonization can be delayed for 8 or more days, but is very common as 60% and 91% of infants may be colonized by 15 and 30 days of hospitalization, respectively. Anaerobic bacteria are far less common (<5%), while gram-negative bacilli can predominate in the gastrointestinal tract. The risk of acquiring microorganisms such as *Klebsiella* spp., *Enterobacter* spp., and others was increased in infants receiving antibiotics for more than 3 days and with an increased duration of NICU stay [28, 97]. The nose, throat, and skin can also be colonized with these potential pathogens. *Candida* colonization can occur in the gastrointestinal tract, skin, and respiratory tract and approximately 19–23% of infants have gastrointestinal tract colonization [39, 73].

Infants most often develop nosocomial sepsis from endogenous flora and are themselves implicated as the major reservoir of potential pathogens that can be transmitted from patient to patient, often via the hands of staff.

Surveillance should be directed at identifying nosocomial infections rather than colonization. If a cluster of infections caused by a particular pathogen is noted, cultures of surveillance cultures should be performed in an effort to detect all babies who are colonized with the outbreak strain. Prompt identification of carriers is necessary to permit cohorting of colonized babies – the first and most important control measure in most epidemics. In addition, identifing specific babies who are at increased risk of developing a nosocomial infection, because this knowledge is important to help the clinician select appropriate empiric antibiotic therapy until definitive culture results are available [29]. However, the purpose of these cultures is not identifying the specific gram-negative bacilli that are colonizing the neonates, but rather to detect the appearance of antibiotic-resistant gram-negative bacilli in the unit. There is necessity of colonization with multi-drug-resistant (MDR) gram-negative rods screening by active surveillance cultures to identify the reservoir for spread of MDR gram-negative bacteria and avoid dissemination between patients [36].

Generally using method for surveillance at birth: bacterial cultures from skin and mucosae (throat, nares, and especially form external ear), they may be helpful in determining of organisms have been transferred from mother to newborn, if obtained immediately after birth. After three day's throat or nare sample, plus culture from skin surface repeating once a week. Stool cultures on the third day in NICU, then weekly. Tracheal aspirates from mechanically ventilated neonates are routinely cultured weekly.

The positive predictive value of surveillance cultures must be very low, since a large number of babies are colonized with a wide spectrum of nosocomial pathogens without ever developing clinical illness. On the other hand, perhaps babies who develop 'normal' bacterial flora, rather than becoming colonized with 'pathogens', have a substantially reduced nosocomial infection risk. This might result in a satisfactory predictive value negative for surveillance cultures [29]. As well as, negative surface cultures do not indicate absence of infection.

Stool

The stool of colonized babies has proved to be the reservoir for continued dissemination of the outbreak strain (gram-negative) in the NICU, usually via the hands of clinical personnel. Transmission occurs so readily because colonized babies generally excrete at least 10^8 gram-negative bacilli per gm of stool continously during their hospitalization. It is recommended weekly stool cultures in all babies who remain in the NICU for 1 week or longer [29].

Endotracheal tube

In many NICUs tracheal aspirates from mechanically ventilated neonates are routinely cultured weekly. Organisms cultured from the endotracheal tube often represent colonization rather than pneumonia. Quantitative cultures from endotracheal tube aspirates may reveal a large number of organisms, and gram stain may demonstrate many white blood cells at times when there are no radiographic or clinical symptoms of pneumonia. Better methods of assessing the microorganisms in the lungs are needed because this is probably a very common site of infection in intubated infants [45, 51, 81, 88].

Tests for bacterial antigens

It is possible to detect the free soluble antigens produced by bacteria multiplying in body fluids such as blood, urine and cerebrospinal fluid, using particle agglutination. They may also detect capsular polysaccharide antigen of GBS, *E. coli* K1, *N. meningitidis* type B, *S. pneumoniae,* and *H. influenzae* type b.

They can be used when antibiotic pretreatment renders culture results unreliable. Recent data suggest the tests have sufficiently poor sensitivity and specificity to render treatment decision [60].

Laboratory evaluation

Leukocyte counts, CRP, ESR, IL-6, IL-8, and CD11b, procalcitonin should be performed if there is evidence of focal infection.

Bacteria

Historically, the predominant organisms associated with neonatal sepsis have changed over time. Gram-negative organisms, especially *E. coli*, were the most common causes of cases of neonatal sepsis reported at Yale University from the late 1940s to the mid-1960s [84]. Nowadays, gram-negative organisms remain the most frequently reported cause of neonatal sepsis in developing countries.

Antibiotic prophylaxis against GBS during labor and delivery has markedly reduced the incidence of GBS sepsis, but an increase in *E. coli* sepsis was noted in some reports [5, 84].

EOS: Generally the data of studies shows the leading role of GBS [11, 31], even nowadays. The second most frequent causative agents are *E. coli* or other gram-negative rods. But in some reports the majority (61%) of EOS was gram-negative organisms, whereas the incidence of GBS sepsis was decreased [84]. It is interesting that *Listeria* spp. just occasionally causes EOS.

LOS: In developed country, a sharp increase in isolation of gram-positive pathogens seems to be relatively constant [31, 83], particularly CoNS reached up to 83% incidency in some reports [31, 54]. Reports from developing countries showed the leading role of gram-negative rods (mainly *Klebsiella* spp., *E. coli*, and *P. aeruginosa*) [17]. The reasons for these differences between developed and developing countries are obscure. Zaidi et al. hypothesized that this may be related

to heavily contaminated environmental sources, high rates of cross-contamination, or the later adoption of appropriate and sophisticated tertiary neonatal care practices in developing countries [104]. Yeasts are also emerging as important nosocomial bloodstream pathogens (9–12%) with possible spread within neonatal intensive care units and thereby posing a potential risk to neonates, including low birth weights [10, 11, 83].

Since the beginning of the era of antibiotics, the prevalent etiologic agents have changed over time as bacterial resistance has developed and new antibiotics have been introduced. A cyclic pattern of pathogen distribution in hospitals has also been described. Gram-positive organisms and gram-negative organisms were shown to have altered in predominance in this report [100].

There is concern that increased use of antibiotics might result in a change in the spectrum of organisms, their susceptibility to antibiotics, or both. Continuous surveillance of the microbiological epidemiology and antimicrobial susceptibilities should be routine for all neonatal intensive care units.

Routes of infection

Transplacental, hematogenous transmission of bacteria is an uncommon route of EOS and occurs primarily with *Listeria monocytogenes.*

The most common route of EOS in preterm and term infants is via an ascending amniotic infection. Members of the maternal genital flora, such as GBS and *E. coli,* the organisms responsible for the majority of cases of EOS, may ascend through the birth canal to the amniotic fluid either through intact amniotic membranes or, more commonly, after rupture of membranes. Once infected amniotic fluid is aspirated or swallowed by the fetus, pathogens may penetrate through immature mucosal barriers, resulting in pneumonia or bacteremia, and may penetrate the blood-brain barrier, leading to meningitis. Thus, prevention and treatment in time of intra-amniotic infection are important steps in preventing preterm delivery and improving neonatal outcome [22]. Unusual pathogens that colonize or infect the vagina or other parts of the mother's genital tract may be the cause of neonatal sepsis due to *Haemophilus influenzae, Streptococcus pneumoniae, Listeria monocytogenes, Salmonella* spp., *Mycoplasma hominis, Candida* spp., and others.

LOS most commonly occurs via horizontal or nosocomial transmission, but it may occur via vertical transmission at birth, leading to colonization and, later, to infection. Skin or mucosal colonization with potential pathogens may be

acquired from the hands of health care workers or family members, from water used in incubator or ventilator humidification systems, or from contaminated fomites such as stethoscopes, which may carry organisms directly from one patient to another. Colonizing organisms may enter the bloodstream through breaks in the skin or mucosa or by gastrointestinal translocation or may be introduced through invasive devices such as vascular catheters, endotracheal tubes, or feeding tubes. Alternately, nosocomial infection may result from infusion of contaminated intravenous solutions (especially lipid-based or high-glucose solutions) or from contaminated formula or breast milk [28].

Gram-positive organisms

Streptococcus agalactiae, Group B Streptococcus [GBS]

The gastrointestinal tract is the natural reservoir for GBS and the source of vaginal colonization. Approximately 10 to 40 percent of pregnant women are colonized with GBS in the vagina or rectum. This colonization can be transient, chronic or intermittent. At least 35% of their infants become colonized. The density of infant colonization determines the risk for invasive disease, which is 40 times higher with heavy colonization. Although only 1/100 of those colonized develop invasive disease due to GBS, > 50% of those present within the 1st 6 hours of life [9]. GBS has a leading cause of maternal chorioamnionitis, puerperal endometritis and neonatal sepsis [14, 38].

The incidence of sepsis in the era before prevention: 5. 1/1000 [75].

In 1996 consensus guidelines aimed at reducing the incidence of GBS EOS were issued by the American College of Obstetricians and Gynecologists, the American Academy of Pediatrics, and the Centers for Disease Control and Prevention (CDC). By incorporating data from multistate, population-based surveillance for early-onset disease into a sample survey of a population of over 600,000 live-born infants, Schrag et al. [75] found that the screening approach was more than 50 percent more effective than the risk-based approach at preventing perinatal group B streptococcal disease. They suggest that a recommendation for universal screening warrants further consideration. In 2002, revised guidelines recommended antenatal culture-based screening as the optimal method for identifying chemoprophylaxis candidates [66].

Following the issurance of the first consensus GBS prevention guidelines, the incidence of EOS decline from 1.7 per 1000 live birth in 1993 to 0.6 per 1000

live births in 1998. After 2002, further reduce incidence to 0.32 0.5 per 1000 live births [14, 31, 65]. In some European countries higher incidence rates have been documented (1.4–5.4/1000 live births).

Transmission of GBS from breast milk, patient-to-patient spread, and colonized nursery personnel has also been reported [50]. The incidence of late-onset disease remained generally stable, averaging 0.34 per 1000 live birth. Analysis of LOS incidence trend since the 1990s suggested that intrapartum chemoprophylaxis does not prevent LOS [76].

The risk of death for preterm infants was more than 3 times that of term infants. Compared to term infants, preterm infants is much more susceptible to invasive GBS disease, in particular LOS and VLOS.

All of the major types of group B streptococci (types Ia, Ib, I a/c, II, III, IV and V) may colonize woman and may cause EOS. Type III strains appear to have special virulence properties for the development of meningitis, and cause >85% of cases of early-onset meningitis and most late-onset group B streptococcal infections [4]. In recent study, among EOS, the most frequent serotypes were Ia (30%), III (28%), V (18%), and II (13%), whereas, among LOS: serotype III accounted for half of all cases; the remainder were predominately serotypes Ia (24%) and V (14%) [65].

Laboratory diagnosis: CDC recommendation that the screening of pregnant women should use optimal microbiologic methods in the antepartum period to attempt to identify all pregnant GBS carriers. The best time to do a GBS screening culture is at 35 37 weeks of gestation. Swab the lower vagina (vaginal introitus), followed by the rectum using the same swab or two different swabs. Cervical cultures are not recommended. Both swabs can be placed into the same container; transport media will maintain GBS viability for up to 4 days at room temperature [66]. For samples sent from the office setting, the use of enriched selective medium and broath appears to have the highest yield and may replace the earlier less sensitive 5% sheep blood agar standard approach [30, 68].

Penicillin is the agent of choice for intrapartum antibiotic prophylaxis, but erythromycin or clindamycin profilaxis is administered for intrapartum prophylaxis among pregnant women colonized with GBS who are at high risk for penicillin anaphylaxis. Investigation data show a high resistance rate to this drug (in one report 32% of GBS were resistant to erythromycin, and 15% to clindamycin) [65]. This observation underscores the importance of performing susceptibility testing.

The presence of GBS bacteriuria in any concentration in a pregnant woman is a marker for heavy genital tract colonization. Therefore, woman with any quan-

tity of GBS bacteriuria during pregnancy should receive intrapartum chemoprophylaxis.

When a rapid GBS screen is required urgently, the direct PCR approach might be used. To maximize the prevention of early onset GBS sepsis for an individual patient, it may be safer to assume that once positive means always positive for subsequent pregnancies.

Because of the risk of emerging resistance and the failure of intrapartum antibiotics to prevent GBS LOS as well as some cases of EOS, efforts to develop a multivalent GBS vaccine are ongoing. A pentavalent conjugate vaccine that included types Ia, Ib, II, III and V could potentially prevent up to 96% of neonatal disease. If ongoing studies fulfill the initial promise, a live recombinant GBS vaccine may be developed that could be given intranasally, reduce bacterial colonization at the mucosal level, and produce both humoral and mucosal immunity.

Staphylococcus aureus

Staphylococcus aureus is a much less common cause of neonatal sepsis in recent decades than at its peak incidence in the 1950s through the 1970s. However, it (and especially meticillin-resistant *S. aureus-MRSA*) can be a special nosocomial pathogen in NICUs. Overcrowding, limited space, inadequate cleansing of the equipment, and initial lack of correct attitude to scrupulous hand washing techniques appear to contribute to the spread of MRSA [1, 2, 38, 90, 92].

Coagulase-negative staphylococci (CoNS)

Although CoNS are common commensal organisms with little pathogenicity in immunocompetent hosts, premature neonates are particularly susceptible to invasive infection. The significance of of CoNS from blood cultures from febrile patients can be difficult, as we discussed at blood culture.

In many institutions CoNS are now the most common cause of all cases of neonatal bacteremia, accounting for > 50% of bloodstream infections [3, 10, 12, 13, 31, 52, 54, 57, 70, 85, 93, 98].

The major species involved in neonatal infection is *S. epidermidis*, which accounts for approximately 50 to 80% of CoNS colonization and 60 to 93% of CoNS bloodstream infection. Other species reported to cause disease in infants include *S. haemolyticus, S. hominis, S. warneri, S. saprophyticus, S. cohnii,* and

S. capitis. The majority of CoNS colonization is acquired nosocomially, predominantly from the hands of health care workers [15, 44, 54, 62, 79].

Neonates with intravascular catheters, particularly those with central vascular catheters which remain in place for prolonged periods, are at high risk for CoNS bacteremia. Another significant risk factor for CoNS septicemia is the use of parenteral nutrition, the administration of intravenous lipid infusions, which provide a growth medium for the organism and frequent use of broad-spectrum antibacterials.

The ability of CoNS to produce slime and biofilms has been linked to increased virulence in preterm infants. Although slime and other virulence factors are important to the pathogenicity of CoNS, several studies did not find evidence of hypervirulent clones of CoNS causing disease in neonates. On the other hand, several studies demonstrated that CoNS sepsis is caused by predominant molecular types which are widely distributed among both neonates and staff, suggesting cross-contamination.

Sepsis with CoNS is often indolent rather than fulminant, although fatalities have been reported. They caused 5–17.3% of all early deaths, respectively [54].

They are often multiresistant to antibiotics. The vast majority (73–100%) of CoNS are resistant to methicillin [14, 70]. NICU infants with suspected late-onset sepsis are typically treated with empiric antimicrobial therapy that often includes vancomycin. The effort, to prevent nosocomial CoNS sepsis in neonates have not been very successful and treating these infections results in a high antibiotic pressure in the NICU, leading not only to an increased risk of antibiotic resistance among CoNS and *S. aureus* but also to the selection of antibiotic-resistant CoNS clones. However, there are national recommendations that vancomycin use in hospitals be restricted because exposure of patients to vancomycin is a risk factor for emergence of vancomycin-resistant enterococci or vancomycin-intermediate *S. aureus*. Currently, there is a lack of consensus among neonatologist on several aspects of diagnosis and treatment of hospitalized neonates with suspected late-onset sepsis.

Enterococcus species

Although accounting for only a small proportion of neonatal sepsis, *Enterococcus* species deserve special mention because of the increasing incidence of neonatal enterococcal sepsis (4–15%) with a mortality rate of approximately 20% in several studies and the emergence of vancomycin resistance among

enterococci. Both *Enterococcus faecalis* and *E. faecium* cause sepsis in preterm neonates, with *E. faecalis* accounting for over 80% of cases. Some studies revealed that the percentage of late onset sepsis caused by enterococci in their study population was high, and this could be partially be explained by the frequent use of cephalosporins as empiric antibiotic therapy throughout the study period [38, 40].

Groups A, C, D, and G Streptococcus species

Species of streptococci other than GBS are infrequent agents in EOS in premature neonates and even less common in LOS. Group A Streptococcus, historically a major agent in puerperal sepsis, has only infrequently been implicated in neonatal sepsis in the last decade.

Listeria monocytogenes

Listeria monocytogenes, a gram-positive bacillus, is a well-known and well-studied neonatal pathogen. Although neonates account for approximately one-third of cases of invasive listeriosis, the organism accounts for less than 5% of cases of LOS in preterm neonates in most studies [47]. The vast majority of cases represent perinatal transmission, although postnatal transmission has been reported [14].

Gram-negative organisms

While gram-negative organisms are responsible for a smaller fraction of neonatal sepsis than are gram-positive organisms, they are associated with the highest mortality [31].

Gram-negative bacteria, particularly members of the Enterobacteriaceae, are normal inhabitants of the intestinal tract. Neonates may acquire early colonization from the maternal gram-negative flora or may develop intestinal colonization after birth with organisms that may subsequently translocate across damaged or immature intestinal mucosa, resulting in LOS, sometimes associated with necrotizing enterocolitis. Other gram-negative organisms such as *Pseudomonas* may be acquired through the respiratory tract, particularly in patients requiring mechanical ventilation.

Gram-negative rods (mainly *Klebsiella* spp.) were the most frequent agents in developed countries 3 decades ago [61], and currently are the most common isolated agents in developing countries [61, 78, 104].

Escherichia coli

EOS with *E. coli* often presents at delivery and is characterized by bacteremia with or without meningitis. Septic shock due to endotoxemia may be a presenting sign. K1 serotype strains cause > 75% of cases of *E. coli* neonatal meningitis, and are the most common and most severe cause of neonatal *E. coli* sepsis. Alternatively, neonates may become colonized with *E. coli* at birth or through contact with colonized caregivers while in the NICU and may develop infection later. Environmental sources include ventilation systems and storage shelves also were identified as source of infection.

While increasing use of intrapartum antibiotics has brought about a decline in the number of cases of early-onset GBS sepsis in neonates, several studies have shown an increase in the incidence of sepsis due to gram-negative bacteria, particularly *E. coli* and particularly among preterm infants [54, 90].

If *E. coli* cause neonatal sepsis, microbiologiest have to check of the antimicrobial sensitivity pattern of this, because of the high and increasing rate of resistance to empirically using antibacterial agents, such as amoxycillin and gentamicin [42]. The growing number of extended-spectrum-lactamase enzyme production is also mentioned in the literature [90].

Enterobacter, Klebsiella, Serratia and Citrobacter spp.

Gram-negative enteric organisms of the Enterobacteriaceae family are common inhabitants of the neonatal intestine which may cause nosocomial sepsis [54] with high mortality. For example incidence of neonatal *Klebsiella* spp. infection in developing countries varies between 2.9 and 12.3 per 100 admissions, with case fatality rate of 18 to 68% [104]. *Enterobacter* spp. frequently isolated in neonatal and paediatric intensive care units, particularly in low birth weight and premature infants. Overcrowding may also contribute to the outbreak of *E. cloaceae* infection. Invasive infections with *Citrobacter koseri*, formerly *C. diversus*, are much more common in neonates than in other patient groups, while *C. freundii* rarely causes disease in neonates.

The possible explanations for the relationship may be poor aseptic practice associated with increased workload and inadequate sterilization of devices [71]. The principal mechanism by which this microorganism is disseminated the cross-transmission by hands or manipulation of nursing staff [85].

Enterobacter spp. and *Klebsiella* spp. showed worrying high rates of multiple resistances [10, 90].

Considering the agressive nature of sepsis caused by these pathogens, particularly in younger infants of low gestational age, empiric treatment against these organisms, especially against those producing ESBL, is worth consideration when managing presumed LOS in VLBW infants [19, 48, 64, 74].

Pseudomonas spp.

Pseudomonas infection is rarely perinatally acquired; however, it is among the more common gram-negative organisms causing nosocomial sepsis in NICU patients. *P. aeruginosa* is commonly thought of as a "water bug", thriving in moist environments such as humidified incubators, sinks, and ventilator circuits. Hands of health care workers have also been identified as an important reservoir [24, 49].

In several studies, *Pseudomonas* had the highest mortality of all organisms causing LOS, with 17 to 75% of patients dying [40, 54].

The incidence of resistant strains has increased with the routine use of drugs such as cefotaxime and ceftazidime. The investigated strains from neonatal infections are very frequently multi-resistant, especially resistant to carbapenems [90], ceftazidime.

Acinetobacter spp.

Acinetobacter species, as special opportunistic causative agents of LOS, showed worrying high rates of multiple resistances [10, 90].

Haemophilus influenzae

Haemophilus influenzae may be vertically transferred from mother to infant at the time of delivery and occasionally causes EOS in preterm infants. Mortality has been reported as high as 90%. Nontypeable *Haemopilus* sepsis has been increasingly identified in neonates, especially premature neonates.

Neisseria gonorrhoeae

Asymptomatic gonorrhea occurs in 5 to 10% of pregnancies, so *N. gonorrhoeae* may be a pathogen as causative agents of EOS.

Anaerobic bacteremia

The role of anaerobes (particularly *Bacteroides fragilis*) remains unclear, although deaths have been attributed to *Bacteroides* bacteremia. In most recent surveys, anaerobes accounted for less than 5% of cases of neonatal bacteremia [41].

Concurrent bacteremia and fungemia, polymicrobial infection

Simultaneous infection with more than one organism is not uncommon in VLBW infants [11, 14, 36, 38, 54].

Treatment of bacterial sepsis

Because of the pure condition of septic-suspicious babies, the antimicrobial therapy is usually begun prior to the isolation of a pathogen and is based upon knowledge of the likely microbes in the particular clinical situation. It should also consider the effects of the use of antimicrobials on the flora of the care unit. The number of antimicrobial agents that can be safely used in neonates is relatively small.

In most centers the mainstay of empiric therapy for *EOS* for both term and preterm infants is ampicillin/amoxycillin and gentamicin, but the emergence of gentamicin-resistant gram-negative bacteria in some centers may prompt the use of other aminoglycosides. In cases of known or suspected sepsis due gram-negative bacteria, and particularly if meningitis is present, addition of a broad-spectrum cephalosporin may be beneficial. The third-generation cephalosporins most used in neonates have excellent CNS penetration in the presence of inflammation; however, extended-spectrum cephalosporins are less active against gram-positive cocci, and their activity against GBS is not as good as that of penicillin or ampicillin. And they have got natural resistance to *Enterococcus* spp., and *Listeria*

spp. For these reasons, ampicillin is added to the cephalosporin when it is used empirically for neonatal sepsis or meningitis. If foul-smelling amniotic fluid is present at birth, therapy for anaerobes (e.g. clindamycin, metronidazole) should be added. Antibiotics may be changed as soon as an organism is identified [14].

For empiric treatment of suspected *LOS* presenting after 3 to 7 days of age, cefalosporins and an aminoglycoside may provide sufficient initial coverage. Most CoNS are resistant to oxacillin, so to every β-lactams, many centers use empiric vancomycin for LOS. If CoNS are suspected (e.g. an indwelling catheter has been in place for >72 h) or are isolated and considered a pathogen, initial therapy for LOS should include vancomycin.

The knowledge of the susceptibility patterns in individual NICUs should be used to guide the choice of antibiotic [21]. If suspected or proven sepsis with gram-negative bacteria warrants initial double-antibiotic coverage for synergy, and an aminoglycoside and a broad-spectrum cephalosporin will the best combination. When empirically use treatment for LOS, consideration should also be given to the possibility of *Pseudomonas* spp. and to *Candida* spp.

The carbapenems imipenem and meropenem also have been used in neonates. Of the two, meropenem is preferred because of considerably less CNS irritation causing seizures. One problem with this class is the high frequency of fungal superinfection.

Tobramycin has greater activity against *P. aeruginosa*. Some gentamicin-resistant isolates will be susceptible to amikacin.

The vast majority of true bacterial pathogens are detected in blood culture within 48 h, and in the absence of other strong clinical indicators of infection, empiric antibiotic use may be discontinued at this time. Once a pathogen is identified, antibiotic coverage should be narrowed based on susceptibility testing. Treatment of bacteremia due to gram-positive organisms has traditionally lasted 7 to 10 days, although shorter courses may be adequate for uncomplicated CoNS bacteremia. Bacteremia due to gram-negative organisms, deep-seated infections, abscesses, meningitis, endocarditis, and osteomyelitis require longer courses (2–3 weeks) of antibiotic treatment.

Typing of isolates is useful as an aid in the control of infection and can exclude sources or reservoirs of infection, identify carriers of infection, determine the prevalence of strains of organisms and identify their patterns of spread and therefore influence preventative programs [100]. Recently, several studies have looked at the use of molecular techniques to define outbreaks and track the pathways of infection. DNA fingerprinting can be very useful in the identification of environmental contamination [16, 23, 24, 35, 43, 46, 67, 87, 96, 103].

Fungi

The incidence of fungal sepsis among preterm infants has increased considerably over the last two decades with increasing survival of smaller, more immunocompromised preterm infants [55]. Fungal sepsis may occur earlier in infants with lower gestational age and birth weight. *Candida* ranges from 2 to 9% of LOS [31, 54, 85] with a mortality rate of 25–60% [35]. Although *C. albicans* and *C. parapsilosis* are the predominant *Candida* species in preterm infants, an increasing number of other *Candida* species have been isolated recently, with azole-resistant *C. glabrata* being one the more common emerging species.

Risk factors for *Candida* spp. sepsis include prolonged (>10 days) use of central intravenosus catheters, hyperalimentation, use of antecedent antibiotics, necrotizing enterocolitis, parenteral nutrition, especially lipids, exposure to H_2 blockers and previous surgery. However, colonization with *Candida* spp. seems to be the most important factor associated with systemic disease. Various reports have indicated that up to 64% of infants develop candidal colonization during their stay in a neonatal intensive care unit and the rectum is the most common site of colonization. Proof of colonization (oropharynx, rectum, skin, bag urine and endotracheal aspirates) may be helpful before culture results are available. One recent study clearly indicate that if an infant in NICU harbours a candidal strain, this strain can be found at multiple sites and can exist for a prolonged period on the basis of results of genotyping. Some VLBW infants are colonized with candidal organisms at birth, suggesting that the acquisitions are from the mothers during labour. Occasionally, the infant can acquire a new candidal strain, but less frequent, then adults during stays in intensive care units.

Because *Candida* may take 2 to 3 days to grow in blood culture, initiation of amphotericin B therapy and removal of the catheter without positive blood or LCF cultures may be life saving.

Amphotericin remains the drug of choice, with a second agent such as flucytosine added for meningitis or persistent infections.

Conclusion

Because of the high incidence and high-risk nature of sepsis among premature infants, "sepsis phobia" is a common phenomenon in the NICU. Nearly all premature infants are exposed to courses of antimicrobial agents. Given the poor outcomes associated with neonatal sepsis despite current optimal antimicrobials

and intensive care, concentrated research efforts should focus on prevention, reliable detection methods, and adjunctive therapies for septic preterm infants.

The knowledge of the frequency and distribution of these infections, the hospital environments and their association with factors that can be controlled is a necessary first step in reducing the risk of nosocomial infection and their associated morbidity and mortality. The best treatment is the prevention, with consistent and agressive hand washing and cleaning procedures in each NICU. Outbreak investigation can be improved with molecular techniques to type and track particular biological strains within a unit.

Traditionally, the function of the laboratory has been to identify pathogens and determine the *in vitro* antibiotic susceptibility. However, it should and must also participate in surveillance and other tasks that aid infection control.

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 53–60 (2009)
DOI: 10.1556/AMicr.56.2009.1.3

BROADENING SPECTRUM OF BOVINE SPONGIFORM ENCEPHALOPATHIES*

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(Received: 19 January 2009; accepted: 22 January 2009)

Until recently the etiology of bovine spongiform encephalopathy (BSE) was considered uniform. The infectious agent was thought to be a single strain of prion (posttranslationally altered form of normal prion protein: PrPSc) retaining its biochemical and biological characteristics during interspecies transmission. However, alternate PrPSc signatures through large-scale screening have recently been detected. In addition, genetic alterations governing susceptibility to prion infection and a mutation (E211K) capable of eliciting spontaneous BSE have been demonstrated. Thus, the spectrum of BSEs have broadened and three PrPSc variants (BSE-C, BSE-H and BSE-L) are now defined. Moreover, a new condition resembling BSE, idiopathic brainstem neuronal chromatolysis (IBNC), has been described that may also turn out to be a prion disease. Since one of the new BSE variants, L-type BSE, proved highly pathogenic detection and further characterization of the new conditions are essential.

Keywords: bovine spongiform encephalopathy (BSE), prion protein (PrP), BSE strain variants (BSE-C, BSE-H and BSE-L), idiopathic brainstem neuronal chromatolysis (IBNC)

Introduction

Transmissible spongiform encephalopathies (prion diseases) constitute a group of lethal, slowly developing infectious diseases that are unique for being as-

* This paper was written to commemorate the 60th anniversary of the foundation of the Institute of Medical Microbiology, Semmelweis University, Budapest, Hungary.

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sociated with an agent comprising of a host protein, the prion protein (PrP), in a posttranslationally altered conformation (PrPSc). PrPSc shows increased resistance to digestion with proteinase K and displays a propensity to form aggregates. This proteinaceous agent, the prion, multiplies without any conventional form of nucleic acid [1, 2].

Prions affect both animals and humans. The most salient prion disease in animals is BSE a condition of cattle. BSE caused an immense, devastating epidemic in the United Kingdom in the 1980s and 1990s involving more than 180,000 animals [3]. Cases of BSE have also been diagnosed across Europe and beyond [4]. BSE proved transmissible to humans [5] and is responsible for variant Creutzfeldt-Jakob disease (vCJD) warranting the introduction of large-scale screening of slaughter animals. In the European Union all slaughter cattle aged 30 months or older and all deceased animals older than 24 months must be tested for BSE [6].

A striking feature of the BSE epidemic was the homogeneity of the agent. While in scrapie, the prion disease of sheep and goat, and in CJD a variety of strains of the agent have been demonstrated [7], BSE seemed to have been caused by a stable form of PrPSc retaining its properties upon multiple transmissions between both identical and diverse species [8]. Individual strains of prion correspond to distinct conformational variants of PrP and display charateristic period of incubation, host range, neuropathological profiles, resistance to digestion with proteinase K and eletrophoretic mobility in PrPSc Western blot assays [9]. The latter is a function of the size, conformation and glycosylation pattern of PrP. Since the BSE outbreak and all the related cases of vCJD were caused by a single strain of prion it turned out to be a surprise when widespread screening of slaughter cattle in various countries subsequently identified "atypical cases" of BSE harbouring novel previously unknown types of the agent.

Diversity of BSE strains in cattle

The first atypical cases of BSE were detected in Italy. Two animals (11 and 15 years old) were diagnosed with an "amyloidotic" form of BSE called BASE in 2004 [10]. The molecular signature of the BASE strain is substantially different from that of "classical" BSE: it commands a proteinase K resistant core of lower molecular mass and the PrPSc glycotype is also dissimilar [10]. In addition, as opposed to "classical" BSE, widespread PrP-amyloid plaques were seen in the supratentorial brain regions while the brainstem was less involved [10].

Concurrently atypical cases of BSE showing a novel type of Western blot signature were diagnosed in France [11]. Though the glycoform patterns proved conventional, the proteinase K resistant core of this atypical strain showed a higher molecular weight than that of "classical" BSE [11].

On the basis of the above findings the strains of BSE have been divided into three groups. "Classical" BSE is called BSE-C, while the strains displaying lower or higher mol. weight unglycosylated proteinase K resistant cores relative to the BSE-C isolate are designated BSE-L and BSE-H, respectively. Additional reports describing cattle with atypical BSE have been published from Germany (both L-type and H-type) [12], Poland (both L-type and H-type) [13], the Netherlands (L-type and H-type) [14], Sweden (H-type) [15], the United States (unclassified) [16], Japan (unclassified) [17] and Belgium (unclassified), [18].

Characteristics of the three types of BSE strains have recently been thoroughly investigated and compared with each other [14]. Apart from the diverse migration profiles differences have been demonstrated in the strains' resistance to digestion with proteinse K and ability to react with various anti-PrPSc antibodies.

At pH values above 7.0 or with increasing proteinase K concentrations the signal intensities of the BSE C-type isolates remained almost unaltered while the intensities of both the L-type and H-type strains declined [14]. At pH 6.5 with a proteinase K concentration of 50 μg/ml the signal for both the L-type and H-type isolates significantly weakened (without any change in the Western blot profile). At pH 8.0 with a proteinase K concentration of 500 μg/ml the signal for both atypical strains practically disappeared. The BSE type-C strain, however, basically retained the intensity of its original signal subsequent both of these treatments [14].

The ability of the various BSE strain types to bind anti-PrPSc antibodies was also investigated. Mainly antibodies used in prion disease diagnostic tests were compared. After digestion with 50 μg/ml proteinase K at pH 6.5 monoclonal antibody SAF32 detected only a single faint band for H-type BSE and no signal for either C-type or L-type strains. Antibody 12B2 bound well to H-type but only weakly to C-type and not at all to L-type BSE. In contrast, monoclonal antibody 9A2 detected bands for all three types of BSE strains [14].

The differing ability of various BSE strain types to adhere to anti-PrPSc antibodies has a bearing on the capability of screening techniques to diagnose atypical forms of BSE. Authors believe that though some cases of atypical BSE could be missed as a consequence of too harsh digestion of the sample with proteinase K or wrong choice of antibody most animals with L-type and H-type BSE will be diagnosed by techniques currently used by screening laboratories [14].

Though the laboratory tests may not pose a problem, taking the sample from the appropriate brain region is a real concern in the diagnosis of atypical BSE. Especially the diagnosis of L-type BSE could suffer as a consequence of inappropriate sampling. While in "classical" (C-type) BSE PrPSc deposits mainly in the brainstem, in animals affected by L-type strains PrPSc resides mostly in the thalamus and olfactory bulb and the brainstem gives only a faint reaction. Since screening is supposed to be performed from brainstem samples this faint reaction could easily be missed and the atypical BSE diagnosis lost [14]. This is a grave concern because preliminary data suggests that L-type BSE represents a human health risk probably even greater than "classical" BSE.

A number of studies investigating the pathogenicity of atypical BSEs have been made. Béringue et al. [19] reported in 2006 that H-type BSE could be successfully transmitted to transgenic mice expressing ovine or bovine PrP. However, the same group subsequently failed to transmit H-type BSE to wildetype mice [20]. In contrast to H-type BSE, L-type BSE proved readily transmissible to a variety of species. Apart from transgenic mice carrying bovine PrP it was transmitted to cattle in which it elicited a prion disease reminiscent but not entirely identical to BSE [21]. Conspicuously, it could be transmitted to wildetype mice without a species barrier [20]. But the real concern is that L-type prion strains readily infect primates and humanized transgenic mice [22]. Transmission studies with transgenic animals suggest that L-type BSE, in contrast to "classical" BSE, is lymphotropic and could pose a more a serious human health risk than "classical" BSE [23]. Casalone et al. [10] observed similarities between the Western blot signature of L-type BSE and that of type-2 sporadic CJD. Though a subsequent report [22] failed to confirm an L-type BSE background for type-2 sporadic CJD it remains to be elucidated whether or not a subset of sporadic cases of CJD is linked to L-type BSE.

In addition, studies with transgenic mice suggest that L-type BSE is a promising candidate for spongiform mink encephalopathy [24] the prion disease of mink which has to date had an obscure etiology.

Apart from possibly posing a serious human health risk L-type BSE is also relevant because it could turn out to be the prion strain from which "classical" (C-type) BSE emerged. Capobianco et al. [25] recently reported that a serial passage of L-type BSE to nontransgenic mice resulted in a neuropathological and molecular disease phenotype indistinguishable from that of "classical" BSE. Authors suggest that the L-type strain has in fact converted to a "classical" BSE strain. Though the proposal is supported by some solid experimental data it remains to be

confirmed. All the more since a scrapie strain (CH1641) showing highly similar Western blot signature to that of the "classical" BSE strain turned finally out to be different [26].

Inherited BSE, genetic background of susceptibility

No case of BSE was observed during the huge BSE epidemic in which inheritance was proved to play a role in the emergence of prion disease, thus, genetic BSE was considered nonexistent. However, with the discovery of atypical BSE two animals have recently been identified in the United States with a mutation capable of eliciting spontaneous prion disease [27, 28]. One of the animals had H-type BSE and carried a mutation at codon 211 of the PrP gene resulting in a glutamic acid lysine substitution (E211K). An analogous mutation at the homologous human codon position (E200K) is well-established to be the most common cause of human genetic CJD. The second animal with identical mutation is the offspring of the first one. It remains symptomless, however, it is under constant surveillance [27].

Though no polymorphisms in the bovine PrP coding sequence rendering the animal susceptible to infection with BSE has to date been observed, two polymorphisms outside the coding region have been demonstrated to influence susceptibility. A 23 bp insertion/deletion site has been detected in the promoter region and a 12 bp insertion/deletion site in intron 1. A significant overrepresentation of the deletion alleles at both mutation sites have been reported in animals with BSE [29, 30].

A number of additional genetic variations have been observed in cattle in the United States [31] and Japan [32], however, the significance of these traits with respect to BSE susceptibility remains to be elucidated.

In addition, a bovine PrP haplotype associated with lower expression of PrP was reported from Germany that proved underrepresented in animals with BSE [33]. Lower expression levels of PrP are supposed to lessen susceptibility to prion infection in all species since the number of available host PrP molecules governs the agent's ability to bring about new conformational conversions.

Idiopathic brainstem neuronal chromatolysis

An additional neurologic condition resembling BSE, idiopathic brainstem neuronal chromatolysis (IBNC), was reported in cattle last year in the United Kingdom [34]. In accordance with BSE, IBNC was characterized by degenerative changes in neurons and marked gliosis. Spongiform degeneration was observed in the midbrain, thalamus, striatum and cortex [34]. Immunohistochemistry demonstrated abnormal labelling for PrP. The pattern of PrP labelling proved different from that seen in BSE. Though PrPSc could not be detected by Western blotting using standard testing procedures weakly proteinase K resistant isoforms of PrP were demonstrated subsequent to mild enzymatic treatment [34]. Authors raise the possibility of IBNC being a novel type of prion disease in cattle. Further studies, including transmission experiments are needed to establish whether or not IBNC is infectious.

Conclusions

BSE like scrapie and CJD turned out to be caused by multiple strains of prion. One of the new strains L-type BSE seems to pose an even greater potential human health risk than "classical" BSE. Since L-type BSE affects different parts of the brain than classical" BSE sampling techniques for BSE screening will be modified.

Genetic traits have been established to govern susceptibility in cattle to infection with BSE. Large scale genetic screening of cattle will determine the susceptibilty of stocks to infection with prions.

A new condition in cattle resembling BSE, IBNC, will further be investigated to elucidate whether or not it is transmissible and poses a health threat to humans.

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 61–69 (2009)
DOI: 10.1556/AMicr.56.2009.1.4

GROWTH, KERATINOLYTIC PROTEINASE ACTIVITY AND THERMOTOLERANCE OF DERMATOPHYTES ASSOCIATED WITH ALOPECIA IN UYO, NIGERIA

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(Received: 21 June 2007; revised: 8 July 2008; accepted: 16 August 2008)

Mycological research was conducted on the mycelial growth, keratinolytic proteinase activity and thermotolerance of dermatophytes associated with alopecia patients in Uyo, Nigeria. The results revealed that *Microsporum* sp. – AP_1, *Epidermophyton* sp. – AP_2, *Trichophyton rubrum* – AP_4, *Trichophyton mentagrophytes* – AP_5 and a yeast *Candida albicans* – AP_3 isolated exhibited variable growth and keratinase activity at different temperatures. *Microsporum* sp. – AP_1 and *T. mentagrophytes* – AP_5 survived heat treatment at 90 °C but exhibited best mycelial growth at 30 °C (with 53.41 mg/50 ml biomass dry weight) and 40 °C (with 61.32 mg/50 ml biomass dry weight) respectively, after incubation for 2 weeks. *Trichophyton rubrum* – AP_4 and *Epidermophyton* sp. – AP_2 could not survive heat treatment at 90 °C but grew better at 40 °C (with 38.52 mg/50 ml biomass dry weight) and 30 °C (with 48.32 mg/50 ml biomass dry weight) respectively, over the same incubation period, while *C. albicans* – AP_3 grew better at 30 °C with 38.7 mg/50 ml biomass dry weight after 2 weeks, but failed to survive at 70 °C. All the isolates except *Candida albicans* – AP_3 survived at 80 °C and exhibited great potential to elaborate keratinolytic enzymes, with *T. mentagrophytes* demonstrating the best potential at 30 °C and 40 °C. Higher temperatures tended to reduce keratinolytic activities and there were significant ($P < 0.05$) relationships between biomass weight and enzyme productivities of all the isolates except *T. mentagrophytes*. This indicates that in some dermatophytes keratinolytic proteinase activity is not a function of cell multiplicity. This plus the high thermostability of the enzymes are important attributes in the consideration of preventive and therapeutic methods against dermatophytes in the tropics.

Keywords: keratinolytic proteinase, thermotolerance, dermatophytes, alopecia

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Introduction

Dermatophytotic fungi commonly known as ringworm fungi are parasitic fungi that infect the skin, hair and nails due to their ability to obtain nutrients from keratinized materials. They depend on their host, which may be an animal (zoophilic) or a human (anthrophilic) and need to spread from one host to another to survive. Anthrophilic dermatophytes are well adapted to live on human skin [1]. Dermatophytes and other pathogenic fungi have been isolated from keratinized materials of animals, birds and humans in many parts of the world [2–4].

Because protease has an important role in the pathogenicity of microorganisms, the ability of dermatophytes to produce protease enzymes including keratinolytic proteinases has been investigated by many researchers [3–6]. Although reports on some species of *Chrysosporium keratinophilum* [8, 9], *Trichophyton mentagrophytes* [10] and *Trichophyton granulosum* [11] are also available, little or no attempt has been made to ascertain the response of keratinolytic proteinase of the ringworm pathogens to different heat treatments. In the present study the effect of different heat treatments on the mycelia growth and keratinolytic proteinase activity of dermatophytes associated with hair samples from alopecia patients in Uyo, Nigeria were investigated.

Materials and Methods

Examination and cultivation of dermatophytes

Direct microscopic examination described by El-Said [4], Sutton et al. [12] and NCCLS [13] was adopted. Samplings of the hair follicles and stratum corneum obtained from suspected infected areas of the patients hair were mounted in 20% potassium hydroxide on a clean glass slide and kept for 20 minutes before examination [4, 14]. The specimens were stained with one drop of lactophenol-cotton blue solution and then examined under the light microscope.

Infected or positive samples were characterized by the presence of fungal hyphae, conidia and budding yeast cells. Samples from ten alopecia patients were examined.

Isolation and characterization of dermatophytes

Infected specimens were deposited on the surface of Sabouraud's dextrose agar plates. Inoculated plates were incubated at room temperature (28± 2 °C) for 21 days and the developing fungal colonies were isolated, purified by repeated sub-culturing and stocked for characterization. Pure cultures of isolates were identified based on the cultural, morphological and biochemical characteristics as described by Sutton et al. [12], Larone [15] and de-Hoog et al. [16].

Determination of the effect of heat treatment on mycelia growth and keratinolytic proteinase activity of the dermatophytes

The inoculum (agar disc bearing the spores and mycelia from an SDA culture) was obtained by using a sterile cork borer of 9 mm diameter. The inoculum of each culture was into plates of keratin substrates derived from scrapings from cow horn. The cow horn scrapings were ground to fine powder defetted for 30 minutes at 37 °C with a chloroform/methanol (1:1 V/V) mixture, stored in water with soap (50% V/V) for 12 h at 42 °C, washed several times with distilled water and dried at room temperature (28± 2 °C) [9, 17]. The resultant powder was used as the basal substrate for the preparation of keratin medium. For the test medium, keratin substrate 10 g was refluxed at 100 °C for 4 h with 500 ml DMSO (dimethyl sulphoxide), cooled and centrifuged at 3000 *g* for 10 minutes. This was followed by precipitation of the supernatant by adding 2 vol. cold acetone (10 °C). After standing at 4°C for 2 h, the mixture was centrifuged at 6000 *g* and the sediment washed in 4-vol water and resuspended in water to give 0.64 mg keratin ml^{-1} as measured by the Lowry method. This procedure has previously, been adopted by Dozie et al. [9].

The test medium, prepared as recommended by Dozie et al. [9] was filter-sterilized and dispensed into 500 ml Erlenmeyer flasks, each containing 50 ml of the fermentation medium. The flasks were inoculated with a suspension of 3×10^6 conidia (determined with a haemocytometer) prepared in phosphate-buffered saline from a week old culture of the isolate grown on Sabouraud's dextrose agar. The inoculated SDA plates were incubated at 20, 30, 40, 50, 60, 80, 90 and 100 °C. The flasks were removed after 14 days of incubation and the mycelia were harvested by filtration through Whatman's filter paper (10 cm). Filtrates from triplicate samples were combined and the arithmetic mean calculated to obtain the dry weight of the isolate.

The five species of dermatophytes recovered from the alopecia-hair samples were screened for their ability to produce keratinase enzymes at different temperatures. Keratinolytic proteinase activity of the dermatophytes was measured by the method of Yu et al. [10] at pH 9. The test mixture containing 1.0 ml of enzyme solution, 4.0 ml of glycine (NaoH buffer (pH 9.0) and 25.0 mg of keratin substance were incubated separately at the prescribed temperature levels for 1 hour).

Results and Discussion

Candida albicans – AP_3, *Epidermophyton* sp. – AP_2, *Microsporum* sp. AP_1, *Trichophyton rubrum* – AP_4 and *T. mentagrophytes* – AP_5 were isolated from the alopecia patient's hair. Among the isolates, Microsporum species was the most prevalent and emerged in 70% of the total samples (Table I). It was followed by *Trichophyton mentagrophytes* and *T. rubrum* with 50% and 40% prevalence rates, respectively. This is almost in agreement with the results recorded in many parts

Table I

Incidence and prevalence of dermatophytes isolated from alopecia patients in Nigeria

	Samples										Total incidence	Prevalence rate (%)
Isolate/Code	1	2	3	4	5	6	7	8	9	10		
Microsporum sp. – AP_1	+	+	–	+	+	–	+	+	+	–	7	70
Epidermophyton sp. – AP_2	–	–	–	–	–	+	+	–	–	–	2	30
Trichophyton rubrum – AP_4	+	+	+	–	+	–	–	–	–	–	4	40
Trichophyton mentagrophytes – AP_5	+	–	+	–	+	–	–	+	–	+	5	50
Candida albicans – AP_3	+	–	+	–	–	–	+	–	–	–	3	30

of the world [3, 4, 18, 19]. The observation is also in harmony with the findings of El-Said (4) and Zaini and Chagan [20] and that *Microsporum* and *Trichophyton* are the two dermatophytes mainly associated with tinea capitis. These dermatophytes have previously been isolated from cases of tinea capitis in Egypt [3], Nigeria [21–24] as well as in some other parts of the world [25, 26]. Although *Epidermophyton* species were encountered in 20% of the cases examined their involvement in ringworm diseases of the hair is rare. *Epidermophyton* has been implicated, however, in cases of invasive dermal diseases in immuno-compromised patients [27]. Similarly *Candida albicans* obtained from 30% of the patient's hair samples has not been implicated in cases of tinea capitis but associated with cases

of cutaneous mycoses and other invasive skin infections such as diaper rashes [28]. The isolates exhibited variable levels of growth (Table II) and keratinolytic proteinase activity (Table III) at different temperatures. *Microsporum* tolerated temperature as high as 90 °C but exhibited the best growth at 30 °C with 53.41 mg/50 ml biomass dry weight after 2 weeks of incubation. *Trichophyton mentagrophytes* also tolerated heat treatment at 90 °C but exhibited best mycelia growth at 40 °C with a biomass weight of 61.32 mg/50ml. *Epidermophyton* sp. and *T. rubrum* could not grow at 90 °C but grew better at 40 °C (with 38.52 mg/50 ml biomass weight) and 30 °C (with 48.32 mg/50ml biomass weight), respectively, while *Candida albicans* preferred 30 °C (with 38.7 mg/50 ml biomass weight) but failed to grow at 70 °C and above.

Table II

Mycelial growth of dermatophytes isolated from Alopecia at different temperature levels
Dry weight (mg/50 ml) after 2 weeks

Isolate	20	30	40	50	60	70	80	90	10
Microsporum sp. – AP_1	22.41	53.41	49.1	47.1	38.41	26.1	18.41	7.62	–
Epidermophyton sp. – AP_2	17.21	33.41	33.52	20.21	14.21	7.82	4.31	–	–
Trichophyton rubrum – AP_4	16.71	48.32	38.33	24.62	28.21	19.42	8.42	–	–
Trichophyton mentagrophytes – AP_5	14.32	51.41	61.32	58.21	59.32	24.36	20.31	44.1	–
Candida albicans – AP_3	24.1	38.7	36.41	30.31	10.12	–	–	–	–

Table III

Keratinolytic proteinase activity of the dermatophytic isolates at different temperature levels
Enzymes (Uml^{-1} h)

Isolate	20	30	40	50	60	70	80	90	10
Microsporum sp. – AP_1	14.31	42.14	65.33	61.32	49.41	33.41	24.21	6.34	–
Epidermophyton sp. – AP_2	14.21	32.41	58.62	54.42	47.34	41.21	13.11	–	–
Trichophyton rubrum – AP_4	10.21	17.41	58.24	48.36	30.00	21.21	14.31	–	–
Trichophyton mentagrophytes – AP_5	7.2	14.33	42.71	50.32	57.41	59.41	68.31	68.42	–
Candida albicans – AP_3	13.4	36.4	33.2	–	–	–	–	–	–

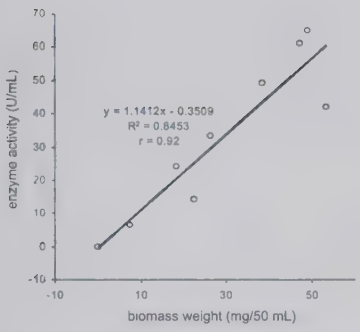
All the isolates exhibited great potential to produce keratinolytic enzymes. The keratinolytic proteinase activities of the dermatophytes varied, however, with the genera and species of dermatophytes screened, and also with the incubation temperature (Table III). *Trichophyton mentagrophytes* demonstrated the best keratinase producing capability, and the enzyme production was generally better at the temperature range between 30 and 40 °C. Higher temperature levels tended to reduce keratinolytic proteinase activities of the dermatophytes. All the dermatophytes but *Candida albicans* exhibited keratiniase activities at temperatures as

high as 80 °C. The yeast failed to elaborate keratinase enzymes at 50 °C and 60 °C despite growth. This implies that factors other than effective nutrient uptake might have contributed to their survival at high temperatures.

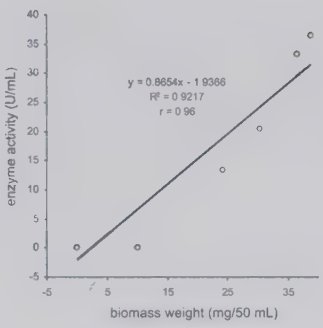
The study has revealed that keratinolytic activity can occur effectively at temperatures above the normal human body temperature of 37 °C. The findings also revealed that the high-temperature short duration (at 60 °C for 30 minutes) procedure commonly adopted by hair dressers in Nigerian saloons may rather enhance the destruction of hair follicles and shafts than elimination of dermatophytes as most of the isolates grew and elaborated the catabolic enzyme at high temperatures. The effects may be more serious because of the de-fattening effect of high temperatures on human hair follicle. Therefore, the low temperature-long duration (40 °C for 50 minutes) process recommended by many Dryer's manufacturers remain the best approach for hair treatment. It is also obvious that despite the positive relationship between biomass weight and enzyme productivity (Fig. 1) of the isolates, a weak or insignificant ($p > 0.05$) relationship was recorded for *Trichophyton mentagrophytes* ($r = 0.42$).

This indicates that in some dermatophytes, keratinolytic proteinase influenced pathogenicity is not a function of biomass production, more so when dermatophytes are dimorphic existing *in vivo* in human tissues as yeast.

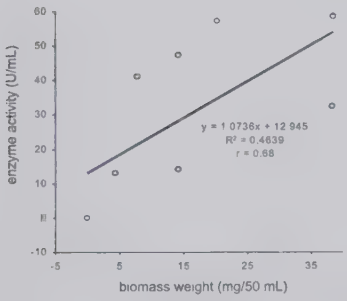
Alopecia is an ailment characterized by absence of hair where it normally grows [29]. Alopecia may be classified as diffuse or localized. It may also be classified as scaring or non-scaring. Dermatophytic fungi have been implicated in both cases [29, 30]. The non-scaring alopecia is normally temporary because there is loss of hair shaft but the follicles are preserved. The alopecia investigated was a temporary one caused by fungal destruction of the hair shaft, sparing the hair follicles. It is commonly associated with the keratinolytic activities of dermatophytes. Proteolytic keratinase is an important virulent factor of dermatophytes especially those involved in dermatophytoses. The present study has shown that ringworm pathogens may contribute to the loss of hair in temporary alopecia patients. It is also apparent that strains of dermatophytes isolated from alopecia infected hair samples can tolerate elevated temperatures with strong ability to produce thermostable keratinolytic proteinase but with variable capabilities to utilize keratin. Heat denatures proteins by causing weak interactions in a protein (primarily hydrogen bonds) in a complex manner. In α-keratin, the cross links stabilizing quaternary structure are disulphide bonds. In the hardest and toughest a keratins such as those of rhinoceros horns, up to 18% of the residues involved in disulphide bonds are cysteines. The α helical structures, which also occur in keratin of human hair, for instance are tough, insoluble structures of varying hardness and flexibility



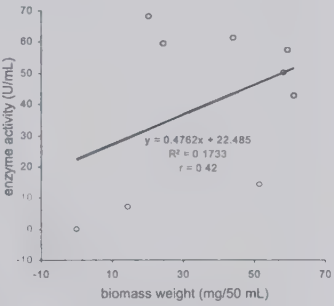
Microsporum sp.



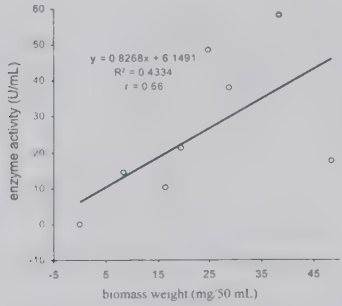
Candida albicans



Epidermophyton sp.



Trichophyton mentagrophytes



Trichophyton rubrum

Figure 1. Relationships between biomass and keratinolytic proteinase activity of dermatophytes isolated from alopecia patients in Nigeria

[31]. This toughness may also be responsible for the ability of dermatophyte's keratinase to resist heat. Their thermostability was the same kinetic parameters as that isolated from horns. So it is important to take in consideration the various methods for protection (e.g. heat treatment of toes in the case athletes foot) from diseases caused by dermatophytes.

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 71–79 (2009)
DOI: 10.1556/AMicr.56.2009.1.5

HEAT RESISTANCE OF DERMATOPHYTE'S CONIDIOSPORES FROM ATHLETES KITS STORED IN NIGERIAN UNIVERSITY SPORT'S CENTER

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(Received: 21 June 2007; revised: 8 July 2008; accepted: 16 August 2008)

The incidence and heat resistance of conidiospores produced by dermatophytes isolated from athlete's kits (canvasses, stockings and spike shoes) stored in Nigerian University Sport's Centre were investigated. *Epidermophyton floccosum, Microsporum oudouinii, Microsporum canis, Trichophyton concentricum, Trichophyton mentagrophytes* and *Trichophyton rubrum* were isolated and their incidence on the athlete's kits varied with the species and type of kits. Among the isolates *T. mentagrophytes, T. rubrum* and *E. floccosum* with 25%, 23% and 20% prevalence rates respectively, were the most common isolates, and are often associated with tinea pedis (athletes foot). Canvasses with the highest incidence of dermatophytes (25 out of 34 fungal isolates) were the most contaminated kits and could serve as effective articles for the transmission of tinea pedis among athletes in Nigeria. The common etiological agents screened, produced asexual spores (conidiospores) that exhibited high resistance to heat treatment at 80 °C. Of the three isolates, *E. floccosum*, with a decimal reduction time (*D*-value) of D_{80} = 4.4 min was the most resistant followed by *T. mentagrophytes* with D_{80} = 4.0 min and then *T. rubrum* with D_{80} = 3.2 min. The spores elimination pattern indicates that increasing the heating duration would decrease the decimal reduction time and possibly denature the fungal propagules but may damage the skin during treatment with hot water compresses. The findings have shown that the use of hot water compresses is palliative but heat treatment especially vapour-heat treatment offers adequate preventive measures if applied for periodic treatment of contaminated kits. However, determining the correct condition for effective decontamination will require detailed understanding of the heat resistance of fungal spores. Otherwise

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treatment of kits with detergent and chaotropic agent such as urea and guanidinium salt is preferred to heat treatment.

Keywords: heat resistance, conidiospores, dermatophytes

Introduction

Diseases of the skin, hair, nail and mucous membranes are the most common of all fungal infections and they show a worldwide distribution [1]. Most of these infections are caused by a group of keratinophilic moulds known collectively as dermatophytes. They cause a complex of diseases known as ringworm (clinical name, Tinea), which affects the keratin in hair, nails, and stratum corneum (top layer) of the skin [1, 2]. Ringworm infections does not create lasting immunity [2], the same type of organism can repeatedly infect a person. An itchy, burning and flaky rash that is circular in shape generally symptomizes the ailments. The skin may also be dry and scaly. Some symptoms of scalp ringworm are flaky circular patches of baldness. Ringworm of the scalp usually begins as small pimple, which becomes larger in size and leaves scaly patches of temporary baldness [3].

Dermatophytes can be transmitted from animals (dogs and cats) to humans and from humans to humans by direct contact with infected person or animal. Ringworms can also be spread through contact with articles such as combs, shoes, canvasses, stockings and clothing or surfaces, which have been contaminated with the fungal spores [4, 5]. One of the most common dermatophytic ailments spread through contact with infected items is tinea pedis commonly referred to as athlete's foot [6]. It is common ringworm associated with athletes and involves the skin of the feet and toes with reduced performance rate. The infection can either be acute or chronic; the re-occurring form of the disease is often associated with fungal infected toenails called tinea unguium [6, 7]. The acute form of the infection most often presents with moist, scaling between the toes with occasional small blisters or tissues. As the blisters break, the infection spreads and can involve large areas of the skin on the foot [8, 9]. The burning and itching that accompany the blister may cause great discomfort that can be relieved by opening and draining of blisters and or applying hot water compresses as commonly practiced by rural Nigerians. This is done with little or no idea about the efficacy of the heat therapy against the etiological agents (*Trichophyton* sp. and *Epidermophyton floccosum*). In a related study on the enzyme activities and thermotolerance of dermatophytes associated with alopecia patients [10] the dermatophytes including *Trichophyton* and *Epidermophyton* species were found to grow and elaborate keratinase activity

at temperature as high as 80 °C. However, the actual effect of the heat treatment on the propagules of the pathogens and extent of tolerance have not been established.

The present study, which investigates the heat resistance of the asexual spores of dematophytes isolated from athlete's kits stored in a University sport centre, is aimed at addressing the subject. The extent of the spore's resistance to heat at 80 °C was determined by establishing the *D*-values (decimal reduction time) of the conidiospores produced by the prevalent dermatophytic isolates associated with the kits.

Materials and Methods

Collection of mycological specimens

Twenty specimens each were collected from athlete's canvasses, spike shoes and stockings in the University of Uyo sports centre. Each sample was collected with the aid of freshly opened Evepon sterile swab sticks (Evepon Industries LTD, Nigeria) and aseptically transferred into 1 ml of sterile physiological saline (0.5%), appropriately labelled and then conveyed to the laboratory for mycological analysis.

Isolation and identification of dermatophytic isolates and other mould isolates

Isolates of the dermatophytic specimens were inoculated on the surface of Sabouraud dextrose agar (SDA) medium using the spread plate technique [10–12]. Inoculated plates were incubated at 28 °C for 3 weeks and the developing fungal isolates were identified based on their cultural, morphological and biochemical characteristics as described by Larone [13], de-Hoog et al. [14], Sutton et al. [15], Barnett and Hunter [16].

Determination of heat resistance of conidiospores produced by the prevalent dermatophytic isolates

The heat resistance of the prevalent dermatophytes obtained from the athlete's kits can be described by the effect of heat on the decimal reduction time (*D*-values) of the fungal spores. The conidiospores of the selected dermatophytes

(*Trichophyton mentagrophytes, Trichophyton rubrum* and *Epidermophyton floccosum*) were cultured on SDA agar (pH 5.5) at 28 °C for 7 days and harvested in phosphate buffer (0.1 mol per litre, pH 5.5) to which Tween 80 (10 g per litre) was added to avoid clotting of the spores. Mycelium and spores were separated by filtration through sterile Kleenex tissues, and the spore's suspension stored at –18 °C. The screw cap-tube technique described by Kooiman [17] and Baggerman [18] was used to determine the *D*-value. For each determination a known amount of conidiospores (determined with the aid of a haemocytometer) was injected into 8 tubes filled with SDA broth (9.9 ml per tube with pH 5.5) as the heating menstruum and previously brought to the temperature (80 °C) desired. The tubes were kept at this temperature for 0, 4, 8, 12, 16, 20, 24, and 28 minutes, respectively, and subsequently cooled in ice water. The numbers of surviving conidiospores were determined on oxytetracycline-yeast glucose agar [19]. The media were incubated at 37 °C and the colonies counted after 10 days.

Results and Discussion

Of the 10 fungal species isolated from the athletes kits only six were major etiological agents of ringworm (Table I). The ringworm pathogens were *Epidermophyton floccosum, Microsporum oudouinii, Microsporum canis, Trichophyton concentricum, Trichophyton mentagrophytes* and *Trichophyton rubrum*. Their incidence on the sport kits varied with the species and type of kits. The most prevalent dermatophytic fungal isolates were *T. mentagrophytes, T. rubrum* and *E. floccosum* with 25%, 23% and 20% prevalence rates, respectively. Canvasses with 34 fungal isolates out of which 25 were dermatophytes were the most contaminated kits followed by stockings and spike shoes with 18 and 15 dermatophytic isolates, respectively.

The heavy load of dermatophytes on canvass may compromise the health and performance rate of the athletes because sportsmen and women routinely use canvasses as relaxation footwear. This implies that longer exposure or contact with the etiological agents of tinea pedis is guaranteed, and agents such as *T. mentagrophytes, T. rubrum, T. concentricum, M. canis, M. oudouinii* and *E. floccosum* encountered in this study have previously been associated with athletes foot [2, 4, 7, 8]. One of the greatest problems hindering the prevention and eradication of ringworm infection is the presence of healthy asymptomatic dermatophyte carriers. Investigation has shown that majority of sports men and women in the University of Uyo had no records or complains of ringworm infections, yet samples ob-

Table I

Fungal species isolated from athletes kits stored at the University of Uyo Sport's Centre

Isolate	Stockings (n = 20)	Canvasses (n = 20)	Spike shoes (n = 20)	Mean prevalence rate (%)
Aspergillus flavus	3 (15)	4 (20)	3 (15)	16.66
Aspegillus fumigatus	2 (10)	3 (15)	1 (15)	10.00
Candida albicans	3 (15)	2 (10)	3 (15)	13.33
Epidermophyton floccosum	4 (20)	4 (20)	4 (20)	20.00
Microsporum oudouinii	1 (5)	2 (10)	1 (5)	6.66
Microsporum canis	2 (10)	3 (15)	– (0)	8.33
Penicillium sp.	2 (10)	– (0)	1(0)	3.33
Trichophyton concentricum	4 (20)	4 (20)	3 (15)	18.33
Trichophyton mentagrophytes	3 (15)	6 (30)	6 (30)	25.00
Trichophyton rubrum	4 (20)	6 (30)	4 (20)	23.33
Total number of fungal isolates	26	34	24	
Number of dematophytic isolates	18	25	15	

n = Number of samples

tained from the sport kits yielded remarkable incidence of dermatophytes. This observation corroborates the findings of Ive [20] and Anosike et al. [21]. Both researchers reported that asymptomatic carriers of dermatophytes might represent a risk equal to symptomatic ones. Therefore, the present findings are vital to the coaches and the University authorities for setting adequate preventive measures necessary to reduce the rate of infection spreading amongst the athletes.

Table II shows the survival pattern of conidiospores derived from *T. mentagrophytes*, *T. rubrum* and *E. floccosum* heated to 80 °C for different periods of time. Relating the log number (log N) of surviving spores with the heating time (minutes), shows that the time required to decrease the number of surviving co-

Table II

Survivability of conidiospores (log number of viable spores) produced by the prevalent dermatophytic isolates at 80 °C

Heat exposure time (minutes)	*T. mentagrophytes*	*T. rubrum*	*E. floccosum*
0	7.973	8.939	7.826
4	7.556	8.531	7.716
8	6.278	6.623	5.505
12	5.477	5.755	4.431
16	4.415	4.792	2.505
20	2.505	2.568	1.778
24	1.568	1.716	1.623
28	1.204	1.079	1.505

nidia of the dermatophytes by 90% at 80 °C were D_{80} = 4.0 min for *T. mentagrophytes* (Fig. 1) D_{80} = 3.2 min for *T. rubrum* (Fig. 2) and D_{80} = 4.4 min for *E. floccosum* (Fig. 3). The figures also show the elimination pattern of conidiospores of *T. mentagrophytes, T. rubrum* and *E. floccosum* at 80 °C increases with increase in the heating duration. This implies that increasing the heating duration resulted in a decrease of the decimal reduction time. From our findings it shows that spores from *Epidermophyton floccosum* with highest decimal reduction time (D_{80} = 4.4 min) was the most resistant followed by *T. mentagrophytes* (D_{80} = 4.0 min) then *T. rubrum* (D_{80} = 3.2 min) when heated at 80 °C. It is relatively obvious that the *D.* values recorded for the dermatophytes are higher than the values recorded for *Aspergillus niger* (D_{50} = 3 min), but much less than D_{77} = 10.3 min, D_{78} = 7 min and D_{79} = 5.5 min recorded for *Aspergillus fischeri* [18]. However, at 80°C conidia from *T. mentagrophytes* and *A. fischeri* exhibited equal resistance (D_{80} = 4 min) to heat.

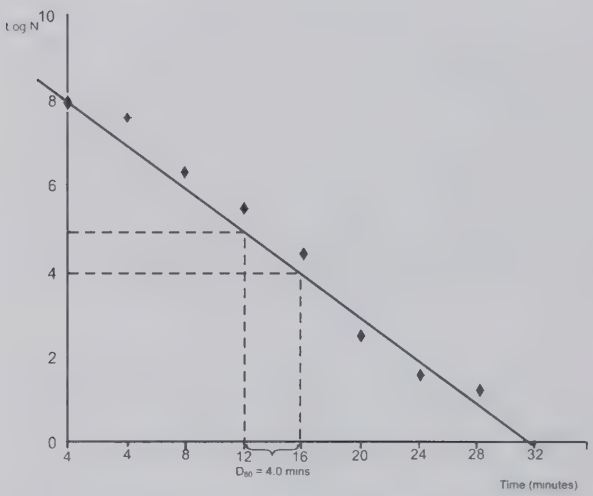

Figure 1. Survival curve of *Trichophyton mentagrophytes* on heating to 80 °C for different periods of time

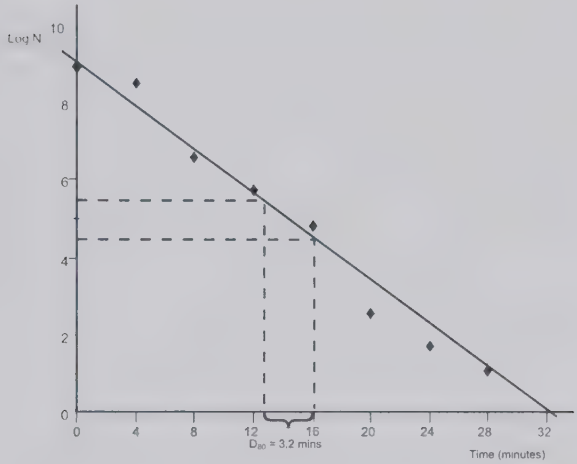

Figure 2. Survival curve of *Trichophyton rubrum* on heating to 80 °C for different periods of time

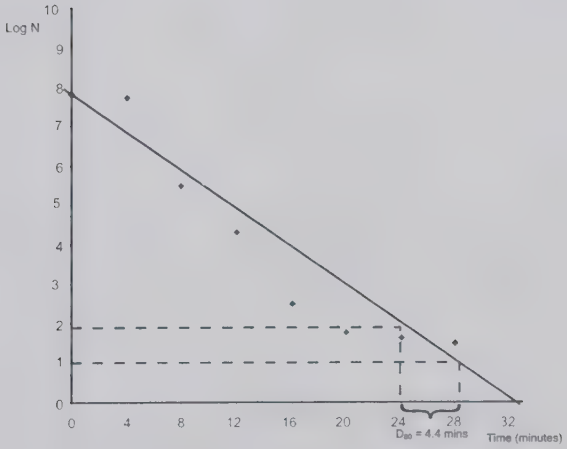

Figure 3. Survival curve of *Epidermophyton floccosum* on heating to 80 °C for different periods of time

This indicates that some species of dermatophytes are more tolerant to heat treatment than the common foodborne mould species of *Aspergillus niger*. However, bacterial endospores with their peptidoglycan component are generally known to show higher heat tolerance [17].

Our findings have shown that sport kits especially canvasses may serve as an effective article for the transmission of Tinea pedis (athletes foot) among athletes in the tropical Nigerian environment. It is also obvious that although ringworm generally responds well to locally applied creams, except for nail and scalp infections (which require oral therapy, [22]), treatment of athlete's foot with hot water compresses is palliative. Such treatment may not denature the propagules of the etiological agents (*Trichophyton mentagrophytes, Trichphyton rubrum* and *Epidermophyton floccosum*). These agents exhibited high resistance to heat treatment at 80 °C, and treatment with higher temperatures that would have denatured their spores could damage the human skin. Therefore heat therapy using very high temperature should be discouraged although it offers an adequate preventive measure if applied for the periodic treatment of contaminated sports kits using vapour-heat treatment. However, the correct condition for effective decontamination will require a full understanding of the heat resistance of the fungal spores at different temperatures, and such treatment should always be preceded by proper washing of the kits. Otherwise treatment of dermatophyte contaminated substances with detergents or chaotropic agents such as urea and guanidinium salt is recommended

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 81–87 (2009)
DOI: 10.1556/AMicr.56.2009.1.6

REVIEW OF MENINGOCOCCAL INFECTION IN CHILDREN AT A UNITED KINGDOM HOSPITAL

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(Received: 22 June 2008; revised: 14 January 2009; accepted: 19 January 2009)

Sixty-nine children were identified and evaluable. Forty-one (60%) presented with hypotension and/or abnormal neurological signs. In 34 (49%) a petechial rash was present on admission. Of note, 13 (19%) had a non-petechial rash, and rash was absent in 19 (28%). Twenty-one (30%) presented with meningism or meningitis. In one child the illness was so mild that the child was discharged prior to making a diagnosis. Five children died (7%). Sixty-three cases (91%) were diagnosed by blood or cerebrospinal fluid culture; these investigations remain the mainstay of diagnosis.

Keywords: *Neisseria meningitidis*, bacteraemia, meningitis, rash

Introduction

Meningococcal disease is an important cause of morbidity and mortality in children despite the availability of broad-spectrum antibiotics. In the United Kingdom (UK) the case fatality for meningococcal septicaemia (in the absence of men-

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1217-8950/$20.00

ingitis) and meningitis were 22% and 2.7%, respectively, in 1995 [1]. The illness can present in a variety of ways [2–4], and is frequently severe [5]. We reviewed the clinical features of invasive meningococcal disease affecting children under 16 years of age presenting to our hospital between 1985 and 1999, with particular emphasis on the type and nature of the rash, severity of illness and clinical outcome.

Materials and Methods

Ethical approval was obtained from the Local Research Ethics Committee. Cases were ascertained by searching microbiology records on the laboratory computer (PathLan), for the years 1985 to 1999 for children less than 16 years of age. A retrospective study of patient case notes was made. The case definition of infection due to *Neisseria meningitidis* was: blood and/or cerebrospinal fluid (CSF) culture yielding growth of the organism, and/or a polymerase chain reaction (PCR) (performed at the Meningococcus Reference Unit, Withington Hospital, Manchester, UK) showing evidence of meningococcal DNA in blood and/or CSF, and/or skin scraping microscopy showing gram-negative diplococci. The following data were collected: age, sex, severe (defined as those patients who presented with shock and/or abnormal neurological signs) versus non-severe presentation, source of referral, presence or absence of fever, duration of illness before presentation, duration of fever following initiation of antimicrobial therapy, presence and type of rash, meningism, headache, microbiology findings, and pre-admission anti-meningococcal antibiotic administration.

Definitions

Shock was defined as having either or both of: a capillary refill time of three or more seconds; hypotension (systolic blood pressure (mm Hg) $< 80 + [\text{age (years)} \times 2]$).

Meningism was defined as having either or both of: neck stiffness; positive Kerning's sign. Meningitis was defined as: CSF culture yielding growth of *N. meningitidis*.

Results

Seventy-five children met the case definition. Six sets of case notes were missing so data was compiled on the remaining 69 (38 females, 31 males) (Figure 1). The male age range was 0–6 years, with a mean of 1.75 years. The female age range was 0–14 years, with a mean of 2.73 years. The ethnic origin of the children was not recorded at case note review.

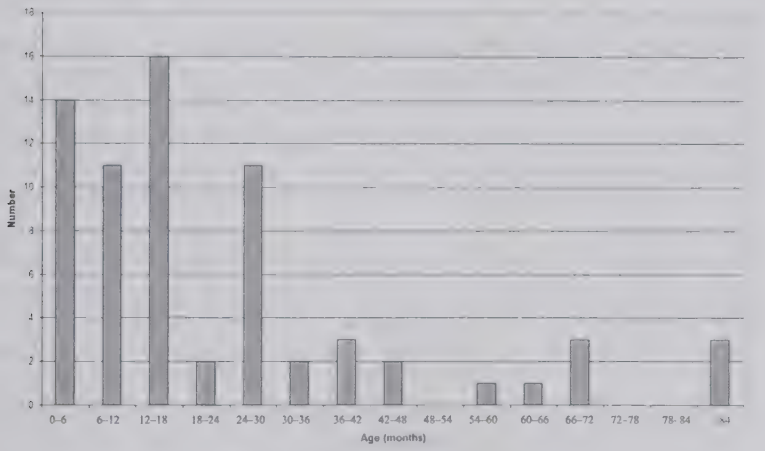

Figure 1. Meningococcal infections by age

Fifty-two (75%) were referred from a General Practitioner (GP), 15 (22%) from the Accident and Emergency Department, and for two cases (3%) the point of referral was not recorded. Forty-one (60%) had a severe, and 28 (40%) a non-severe presentation. Five children died. The duration of symptoms prior to hospital admission ranged from 30 minutes to seven days with a mean of 39 hours. Fifty-six (81%) presented with a febrile illness (temperature > 38 °C). The mean duration of fever following initiation of anti-microbial therapy was 3.6 days (0 to 18 days). In 34 (49%), a petechial rash was identified on admission, in 13 (19%) a non-petechial rash was seen, in 19 (28%) rash was absent and in three (4%) it developed following hospital admission. Of the 32 patients who had no rash or a blanching rash, 16 were bacteraemic, 12 had severe presentation, and 21 had meningitis. Three of the 19 patients without rash had no evidence of meningitis me-

ningism. Five of the 13 with non-petechial rash had no evidence of meningitis/meningism.

Twenty-one (30%) presented with meningism and 48 (70%) without. One patient (1.4%) developed meningism after admission. Headache was documented in 23 (33%) of those over 3 years of age. In 19 (28%), the illness was preceded clinically by a history of an upper respiratory tract infection. One child was assessed at the hospital and sent home with a diagnosis of viral illness. He presented the following day with abnormal neurological signs and was managed successfully.

Blood cultures yielded *N. meningitidis* in 36 (52%) of cases and were negative in 31 (45%). In two (3%) patients, blood cultures could not be traced. Fifty of 61 (82%) CSF cultures grew *N. meningitidis,* and in 27 of these, it was the only diagnostic test indicating meningococcal disease. Blood PCR was positive in 6 of 8 blood specimens tested, and one of one CSF specimen tested. In one child, the CSF culture yielded a diagnosis in the absence of both a growth from blood culture and a positive blood PCR. Blood PCR was the only positive test in two of 15 (13%) children presenting after the test became available in the UK. In three, the diagnosis was made only by positive microscopy of skin scrapings (in only one of these was a blood PCR performed, which was negative).

N. meningitidis group B accounted for 36 (52%), group C for 24 (35%), and group A for one (1.4%) of cases. In 11.5% the strain could not be identified.

Only two (3%) had received intra-muscular penicillin before being seen by a paediatrician. One had a petechial and one a non-petechial rash. Four (6%) children were transferred to the regional tertiary care unit for intensive care. All of the five children who died were severely ill at presentation (three died locally and two died at the tertiary care centre). None of these children had received pre-hospital intra-muscular antibiotics even though three had been seen by a GP and had petechial rash on admission to hospital.

Discussion

This review provides a recent picture of the clinical spectrum of illness due to *N. meningitidis* presenting to a non-specialist acute hospital in the United Kingdom, prior to introduction of the group C meningococcus vaccine. The illness was severe (hypotension and/or abnormal neurological signs) in 60%, with a mortality of 7%. This is consistent with a fatality rate of 5.6% for England and Wales [6].

The proportion with petechial or purpuric rash on admission in this series (49%) is in keeping with the only published series in children we can find which reports on this [4], although one prospective study on the features of haemorrhagic rash in systemic meningococcal disease (which included non-laboratory confirmed cases) showed 47 of 57 (82%) cases in the 0 12 year age group had haemorrhagic skin rash [7]. This paper may have over-represented haemorrhagic rash, as some of these cases may not have been meningococcal. It is possible that the nature of our study (using laboratory investigation based case definition) may have underestimated the number of children presenting with rash, as some of these cases might have received pre-admission antibiotics rendering laboratory investigations negative. Importantly, the finding that rash was absent or non-petechial/purpuric in nearly half of admissions should be noted, as petechial/purpuric rashes are often used as indicators of severe illness requiring hospital admission. It is often considered that rash may develop some time after other symptoms and signs [8].

The fact that, of the five deaths, all had severe illness at presentation supports the need for prompt referral to hospital, before the illness has progressed to this stage. In eight children there were no clinical pointers to meningococcal disease (three had no rash and no meningism/meningitis, and five had non-petechial rash and no meningism/meningitis). Microbiological investigation allowed appropriate and successful therapy to be instituted.

It was not the purpose of this study to review the microbiological methods of diagnosis in detail, and the PCR test only became available at the very end of the study period. However, our findings support that culture of blood and CSF, microscopy of skin scrapings, and PCR testing of blood and CSF, are all useful investigations. CSF analysis may be of use to make a diagnosis of meningococcal infection (and meningitis). Of note, our study shows that in many cases the CSF culture yielded a growth of *N. meningitidis* when the blood culture was negative. In our study there were too few children evaluable (only eight) post availability of the meningococcus PCR test in the UK to draw any conclusions as to how often the CSF culture may yield a diagnosis when blood culture and blood PCR fail to diagnose meningococcal disease. However, with present turnaround times for PCR testing of blood and CSF specimens, the CSF white cell count and culture yield useful and timely information to aid management. The role of lumbar puncture (LP) has been discussed recently [9]. We believe CSF analysis remains an important investigation to obtain a microbiological diagnosis, and that LP should be performed unless there is a specific contra-indication.

It is well described in the literature that some cases of meningococcal disease may present with benign illness [10–12], and in this study one child was assessed and discharged home on the day of admission before the blood culture yielded a growth of meningococcus. He re-presented the following day and was successfully treated.

It is of concern that so few of the children who had been seen by a medical doctor had received intra-muscular penicillin prior to admission to hospital. The Chief Medical Officer (CMO) of England wrote to chief executives of health authorities on 19 Jan 1999 [13], highlighting the importance of GPs administering intra-muscular benzylpenicillin prior to hospital transfer, although in this study only two had received such therapy (both were successfully treated). Three children who died had been seen by a GP prior to hospital admission, and had not received intra-muscular penicillin, two of these presented after the issue of the CMO letter [13]. There is reasonable evidence that such therapy improves outcome [14, 15].

It should be noted that meningococcal disease may present variably, and initial clinical judgement can be misleading especially in less severe cases. This fact indicates that, though the presence of petechial rash suggests the diagnosis, its absence does not rule out meningococcal disease and the ultimate diagnostic tool is a good clinical examination with a high suspicion for this potentially fatal disease. Because of the media publicity, people are well aware of the concern about petechial rashes. We suggest that they should be made aware of the other modes of presentation of meningococcal disease.

Acknowledgement

The authors have declared no funding sources.

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 89–99 (2009)
DOI: 10.1556/AMicr.56.2009.1.7

THE PREVALENCE OF EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* AMONG CLINICAL ISOLATES FROM A GENERAL HOSPITAL IN IRAN

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(Received: 25 November 2008; revised: 10 January 2009; accepted: 19 January 2009)

This study was conducted at a 900+ bed general teaching hospital, from May to September 2007, in Iran. The aim of this study was to determine the prevalence of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* and their antimicrobial pattern. The Kirby-Bauer disk diffusion method and the phenotypic disk confirmatory test were performed for each isolate. The total of 206 isolates including 106 *E. coli* and 100 *K. pneumoniae* were collected of which 122 isolates (59.2%) were ESBL positive. The prevalence of ESBL-producing strains was 59.2% (122 206). All the isolates were susceptible to imipenem. Among the ESBL-producing isolates, the sensitivity was from 3.3% to 61.5% for ampicillin to aztreonam. From female isolates (136), 59.5 % and from male isolates (70), 58.6% were ESBL-producers. Ratios of isolates from hospitalized patients to out-patients were 94.28 in the ESBL-producing group. The number of ESBL-producing isolates according to the isolation sites showed a significant difference between ESBL-producers and non-producers in blood samples ($P < 0.05$). This study shows that the prevalence of ESBL strains in Iran is high. It seems necessary for clinicians and medical community personnel to be fully aware of ESBL-producing microorganisms.

Keywords: clinical isolate, phenotypic confirmatory test, extended-spectrum beta-lactamase (ESBL), *Escherichia coli, Klebsiella pneumoniae,* prevalence

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Introduction

Beta-lactamase production is the most important mechanism for resistance of Gram-negative bacilli to beta-lactam antibiotics [1, 2]. *Escherichia coli* and *Klebsiella pneumoniae* are among the bacteria which produce extended-spectrum beta-lactamase (ESBL) [3]. ESBL production causes resistance to multiple antimicrobial agents [4, 5]. The incidence of ESBL-producing strains among clinical isolates has been steadily increasing over the past years resulting in limitation of therapeutic options [6–9]. The frequency of ESBL-producing organisms differs significantly by geographic location [2]. ESBL-producing bacteria are usually found in those areas of hospitals where patients are critical and antibiotic use is frequent, particularly intensive care units, surgical wards, neonatology wards and oncologic wards [10, 11]. Patients are most likely to be affected if the hospital or intensive care unit stay is prolonged and following treatment with multiple courses of antibiotics, especially extended-spectrum cephalosporins [2].

Despite the difficulties in laboratory detection of ESBL, it is important to identify the prevalence of ESBL production among *E. coli* and *K. pneumoniae* in regional hospitals. There are some recent reports on the prevalence of different phenotypes of ESBL strains in Tehran (Iran) hospitals [12, 13]. The aim of this study was to determine the prevalence of ESBL production and antimicrobial pattern of *E. coli* and *K. pneumoniae* isolates from patients at Imam Reza General Hospital, Mashhad, Khorasan Razavi Province, I. R. Iran over a period of 6 months.

Materials and Methods

Clinical isolates

This study was conducted on 206 clinical isolates of *E. coli* and *K. pneumoniae* which were collected by the microbiological laboratory at Imam Reza Hospital, a 900+ bed general teaching hospital, from May to September 2007. All isolates were collected from different patients who were either hospitalized in various types of wards or admitted as out-patients. Isolates were identified by standard microbiological methods [14]. The isolation sites of bacteria and gender of patients also recorded.

Susceptibility tests and confirmation of ESBL production

Detection methods for ESBLs usually include an initial screening test for reduced susceptibility to a range of extended-spectrum cephalosporins such as cefotaxime, ceftriaxone, ceftazidime and aztreonam. This is then followed by a phenotypic confirmatory testing where the ability of a beta-lactamase inhibitor, usually clavulanic acid, to reduce the resistance is tested.

The 206 isolates included in this study were from urine, blood, wound swab, vaginal swab, urethral discharge, eye swab, plural fluids, and cerebral spinal fluid (the last 5 samples considered as others).

The Kirby-Bauer disk diffusion method using Mueller–Hinton agar and commercial disks (PadTan Teb,Iran) were used for susceptibility tests [15]. The following antibiotic disks were used: imipenem (10 μg), ampicillin (10 μg), aztreonam (30 μg), cotrimoxazole (5 μg), gentamicin (10 μg), ceftazidime (30 μg), ceftriaxone (30 μg) and ciprofloxacin (5 μg). The antibiotic disks impregnated culture plates were incubated at 37 °C for overnight. The diameter of the zone of inhibition was measured and recorded as resistant or susceptible according to the CLSI [16]. The phenotypic disk confirmatory test was performed as the standard disk diffusion assay for each isolate as recommended by CLSI. In this test, microorganism was swabbed onto a Mueller Hinton agar (Himedia, India) plate. A susceptibility disk containing cefotaxime/clavulanate (30/10 μg) or ceftazidime/clavulanate (30/10 μg) disks were placed in the center of the plate with other disk containing cefotaxime (30 μg) or ceftazidime (30 μg) at variable distance (20 -30 mm) center to center from the cefotaxime/clavulanate (30/10 μg) or ceftazidime/clavulanate (30/10 μg). Clavulanate enhancement of the diameter of the inhibition zone around either the antibiotic disk by at least 5 mm was regarded as evidence for the presence of ESBL.

Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Science version 11.5. Contingency table analysis was carried out by κ^2 test, Fisher's exact test if appropriate. A P-value <0.05 was considered statistically significant.

Results

Susceptibility tests results

During the study period, 106 isolates of *E. coli* and 100 isolates of *K. pneumoniae* were collected (Table I). There were 122 patients from whom ESBL-producers *E. coli* or *K. pneumoniae* were detected. Of these isolates, 61 were *E. coli* and 61 were *K. pneumoniae*. The prevalence of ESBL-producing strains was 59.2% (122/206). Table II shows the results of susceptibility test. Using the disk diffusion method all the isolates was susceptible to imipenem. Among the ESBL-producing isolates, the sensitivity was (%) 3.3, 4.9, 6.5, 8.2, 32.8, 42.6, 44.3 and 61.5 for ampicillin, ceftriaxone, cefotaxime, ceftazidime, cotrimoxazole, ciprofloxacin, gentamicin and aztreonam respectively.

Table I

Frequency of bacteria producing ESBL among the isolates

Organism	Number of isolates	ESBL(+) N (%)	ESBL(–) N (%)
Escherichia coli	106	61/106 (57.5)	45/106 (43.3)
Klebsiella pneumoniae	100	61/100 (61)	39/100 (39)
Total	206	122/206 (59.2)	84/206 (40.8)

CLSI confirmatory tests for ESBLs

All the isolates were tested with the CLSI phenotypic confirmatory test for ESBLs. 122 isolates (59.2%) gave positive results, indicating of classical ESBLs by these isolates. From ESBL positives, 61 isolates of *E. coli* (57.5%) and 61 isolates of *K. pneumoniae* (61%) were ESBL positive (Table I).

Table III shows the frequency of isolates according to gender and out or in-patient distribution. 136 isolates were from females and 70 from males. From female isolates, 59.5 % and from male isolates 58.6% were ESBL-producers, with no significant difference between female or male for ESBL-producers ($P > 0.05$). In another word, male to female ratios were 41/81 in ESBL-producing group and 29/55 in ESBL-non-producing group (Table III). Also there is significant difference between in or out-patients isolates in ESBL production ($P < 0.05$). From total of 122 ESBL-producer isolates, 94 (64.4%) isolates were from in and 28 (46.7%)

Table II

The antimicrobial susceptibility patterns of ESBL-producing and non-producing strains to various antibiotics

Antibiotic	ESBL-producers	Non ESBL-producers	Total	*P-value
	N = 122 (%)	N = 84 (%)	N = 206 (%)	
Imipenem	122 (100)	84 (100)	206 (100)	–
Ampicillin	4 (3.3)	12 (14.3)	16 (7.8)	<0.05
Aztronam	75 (61.5)	55 (38.5)	130 (63.1)	>0.05
Cotrimoxazole	40 (32. 8)	57 (67.8)	97 (47.1)	<0.05
Gentamicin	54 (44.3)	60 (71.4)	116 (56.3)	>0.05
Ceftazidime	10 (8.2)	79 (94.0)	89 (43.2)	<0.05
Ceftriaxone	6 (4.9)	72 (85.7)	78 (37.9)	<0.05
Cefotaxime	8 (6.5)	76 (90.5)	84 (40.8)	<0.05
Ciprofloxacin	52 (42.6)	82 (97.6)	134 (65.0)	<0.05

* Fisher's Exact Test

Table III

Frequency of isolates (ESBL-producers and non-ESBL-producers) according to gender and out or in-patient distribution (of the patients)

Number of isolates	ESBL(+)	ESBL(–)	*P-value
Hospital isolates	94/146 (64.4)	52/146 (35.6)	
Out patient isolates	28/60 (46.7)	32/60 (53.3)	
Female	81/136 (59.5)	55/136 (40.5)	<0.05
Male	41/70 (58.6)	29/70 (41.4)	
* Fisher's Exact Test			>0.05

from out-patients with significant difference (P <0.05). In this study, hospital isolates to out-patient isolates ratios were 94/28 in ESBL-producing group and 28/32 in ESBL non-producing group.

Table IV shows the distribution (number and percentage) of ESBL-producing isolates according to the isolation sites. The number of ESBL-producing isolates according to isolation sites of bacteria was as follows: 48 isolates from blood, 27 from urine, 33 from wound, and 14 from others. There was significant difference between ESBL-producers and non-producers in blood samples (P <0.05), but not in urine, wound and other samples (P >0.05).

Table IV

Number and percentage of ESBL-producing and non-producing isolates from different specimens

Isolation site	ESBL(+) (%)	ESBL(–) (%)	*P*-value*	*P*-value**
Urine	27/45 (60)	18/45 (40)		>0.05
Blood	48/63 (76.2)	15/63 (23.8)		<0.05
Wound	33/60 (55)	27/60 (45)	<0.05	>0.05
Others	14/38 (36.8)	24/38 (63.2)		>0.05

* Pearson Chi-square
** 2 proportional test

Discussion

Resistance to beta-lactam antibiotics, especially extended-spectrum cephalosporins and other antibacterial agents, among clinical isolates of Gram-negative bacteria is increasing worldwide [17–19]. Reports of clinical failure and different infections due to ESBL are emerging [20–22]. The main goal of this study was to assess the prevalence of ESBL-producing *K. penumoniae* and *E. coli* and to evaluate co-resistance among clinical isolates in a general hospital.

In this study the prevalence of ESBL-producing isolates was 59.2% (122/206). The rates was significantly higher in hospitalized patients than non-hospitalized ones ($P < 0.05$) (Table III). The prevalence of ESBL in current study is higher than the previous report from two medical centers in Tehran, the capital city of Iran [12]. In their report on frequency of ESBL-producing *K. pneumoniae* from different specimens of patients they showed 44.53% of total isolates (128) were ESBL-producers.

Using the criteria of National Committee for Clinical Laboratory Standards the prevalence of ESBL is probably underestimated [23]. The rate of ESBL varies in different countries. Bradford (2001) report indicates the rate of ESBL of 45.5% in Latin America, 24.6% in Western Pacific, 22.6% in Europe, 7.6% in United States and 4.9% in Canada [24]. In the Netherlands, a survey of 11 hospital laboratories showed that < 1% (6 out of 767) of *E. coli* and *K. pneumoniae* strains possessed an ESBL [25]. However, in France as many as 11.4% of *K. pneumoniae* and 47.7% of *E. aerogenes* were found to be ESBL-producers [26]. Report on the great diversity of ESBL and the prevalence of clinical isolates of ESBL-producing *E. coli* and *K. pneumoniae* indicate that this is an important problem in Spain [27]. Reports on the prevalence of ESBL-producing *E. coli* and *K. penumoniae* in other countries are different. In Ozgunes et al. (2006) report the prevalence of ESBL-

producing isolates of *K. penumoniae* and *E. coli* were 47% and 12%, respectively [28]. These rates were 36.1% in hospitalized and 13.3% in non-hospitalized group. Navon-Venezia et al. (2003) studied 438 isolates which 42.5% (186) were ESBL-producers [29]. Menon et al. (2006) report on Enterobacteriacea family indicates that 20% of isolates were ESBL-producers [30]. Khurana et al. (2002) report on 233 isolates indicates that 26.6% were ESBL-producers and 38.5% *K. penumoniae* and 24.7% *E. coli* [31]. Kumar et al. (2006) work on 100 isolates of Enterobacteriacea showed that 19.8% of isolates were ESBL from which 63.7% *E. coli* and 14% *K. pneumonia* [32]. Kjerulf et al. (2008) reported that the frequency of ESBL-producing *E. coli* and *Klebsiella* isoiates was low in the Copenhagen area of Denmark (0.8%) [33].

The prevalence of production of ESBL among our isolates were mainly from blood culture ($P < 0.05$) (Table IV). This was not significant ($P > 0.05$) for other samples, urine and wound. The majority of the ESBL-producers among our isolates were *K. pneumoniae* (61%) and this is similar to *K. pneumoniae* rate in other findings from Kingdom of Saudi Arabia (42%) [34], United States (48%) [35] and Argentina (48%) [36], where *K. pneumoniae* was the most common ESBL-producers.

Our results show that all isolates either ESBL-producers or non-producers were susceptible to imipenem (Table II) which is in agreement with the other reports [37, 38]. ESBLs do not confer resistance to carbapenems such as imipenem, and this class of antibiotics is considered the therapy of choice for invasive infections caused by ESBL-producing organisms [39, 40]. Also the majority of ESBL positive isolates exhibit decreased susceptibility towards third generation cephalosporins ($P < 0.05$) and high co-resistance to other antimicrobial agents (Table II). The emerging resistance may be attributed to the indiscriminate use of these antibiotics.

Plasmid containing genes encoding ESBLs frequently transport the resistance genes to other antimicrobial drugs such as aminoglycosides, cotrimoxazole and special fluoroquinolones [41]. Widespread fluoroquinolone resistance among ESBL-producing strains has been increasing up to 40–50% [42, 43]. Paterson et al. (2000) found that 60% of ESBL-producing *K. pneumoniae* strains were co-resistant to ciprofloxacin [44]. Although the exact cause of fluoroquinolone resistance in ESBL-producing strains remains to be unknown. This resistance was reported to be due to mutations of *gyr A* genes or porin loss. The ESBL-producing *K. pneumoniae* and *E. coli* strains are more frequently resistant to aminoglycosides and cotrimoxazole than non-ESBL-producers [41]. Goyanes et al. (2007) reported high rates of co-resistance with fluoroquinolones, cotrimoxazole and aminoglyco-

sides in ESBL-producing *E. coli* and *K. pneumoniae* strains [45]. Based on multi resistance in ESBL-producing Gram-negative bacteria, in present study co-resistance to above-mentioned antibiotics were evaluated (Table II). Our results confirm high rates of co-resistance with these antibiotics. Also this study showed imipenem activity has not been affected by ESBL production which has important clinical implications.

Several studies have reported that inappropriate initial antimicrobial therapy for bacteremia caused by ESBL-producing *E. coli* or *K. pneumoniae* is associated with significant higher mortality rate than when initial therapy involves an active agent [45]. Therefore, it is recommended in areas with a high incidence of Enterobacteriaceae resistant to third generation cephalosporins to identify the patients with sever infections who should receive empirical treatment with an agent active against these organisms, and only carbapenem seems to be an appropriate empirical alternative when a sever infection due to a third generation cephalosporins resistant microorganisms is suspected.

Though data regarding the prevalence of ESBL strains in Iran is limited, but it seems higher than the results in similar studies in other countries. This could be because of the overuse of third generation cephalosporins. However, it seems necessary for clinicians and medical community to fully aware of ESBL-producing microorganisms. Therefore, microbiological surveillance on ESBLs at both local and national levels and computerized antibiotic control program could play a major role in establishing more effective antibiotics policies.

Acknowledgements

Funding: This research project was supported by the Vice Chancellor for Research, Mashhad University of Medical Sciences, Mashhad, IR Iran (grant No. 85411).

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Acta Microbiologica et Immunologica Hungarica, 56 (1), pp. 101–114 (2009)
DOI: 10.1556/AMicr.56.2009.1.8

BIOTRANSFORMATION OF L-TYROSINE TO TYRAMINE BY THE GROWING CELLS OF *LACTOCOCCUS LACTIS*

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(Received: 31 December 2008; accepted: 22 January 2009)

The potential of pyridoxal-5-phosphate dependent tyrosine decarboxylase (E.C. 4.1.1.25) of *Lactococcus lactis* was explored for the biotransformation of L-tyrosine to tyramine. Maximum bioconversion of L-tyrosine to tyramine was achieved in tyramine production medium (pH 5.5) at 30 °C after 16 h of incubation with 0.2% L-tyrosine. The yield of tyramine was found to be 11.8 μg/mL by the growing cells of *L. lactis* at shake flask level. Growth medium and different physico-chemical parameters to maximize the biotransformation of L-tyrosine to tyramine were optimized and yielded 1.9-fold increased synthesis of tyramine.

Keywords: *Lactococcus lactis*, biotransformation, tyrosine decarboxylase, tyrosine, tyramine

Introduction

The tyramine (decarboxylated product of L-tyrosine) is a naturally occurring amine which acts as an indirect sympathomimetic in mammals. Tryamine act as a biochemical precursor for dopamine [1, 2] and octopmine which has been shown to play major role in invertebrate nervous systems as a neurotransmitter, neuromodulator and neurohormone [3]. The primary established action of tyramine is increased release of norepinephrine which in turn increases forskolin-me-

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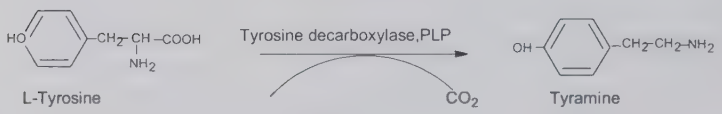
diated cAMP generation and beta adrenergic mediated lipolysis, thus is being used in various health supplements for reducing body fat [4]. Tyramine and Vanadate both has been found to act as insulin mimicker that stimulates glucose transport and can be used as effective therapeutic agent in Diabetes Mellitus type II [5]. It has been discovered and demonstrated that tyramine and its derivatives in cosmetic population reduces the melanin production in an effective manner [6]. Despite of its diverse applications, some individuals being treated with MAO inhibitors, tyramine produces some undesirable health problems such as cardiovascular arrest/stroke [7].

Reaction catalyzed by tyrosine decarboxylase



Since it has only been recognized as a neurotransmitter about a decade ago, comparatively limited reports are available about tyramine [8]. Although the industrial production of tyramine has been limited to chemical synthesis process, the use of enzymatic system of tyrosine decarboxylase has considerable potential. The decarboxylation has been reported among many *Lactobacillus* species but *Lactobacillus curvatus* appeared as the main producer. However some strains of *Lactobacilllus bovaricus, Lactobacillus brevis, Lactobacillus paracasei* and *Lactobacillus sakei* and *Lactococcus lactis* were also reported to produce tyramine from L-tyrosine [9–11]. Keeping in view the scientific utility of microbial tyrosine decarboxylase in the synthesis of potentially important intermediate like tyramine this study reports the biotransformation of L-tyrosine to tyramine using growing cells of *Lactococcus lactis* at shake flask scale.

Materials and Methods

Chemicals and microorganisms

All the chemicals used were of analytical grade, however, the media components were procured from Hi-media, Mumbai, India. The HPLC grade solvents were used in HPLC analysis. The *Lactococcus lactis* BTS-5 was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla-5. Pure cultures of the strain were established by repeatedly streaking on tyramine production medium agar plates until a single discrete colony was obtained. Pure cultures were maintained on tyramine producing medium slants and stored at 4 °C. Repeated sub-culturing was also carried out.

Production medium and preparation of growing cells of L. lactis

L. lactis was grown in a tyramine producing medium [26] containing (g/L) meat extract (8.0), tryptone (5.0), yeast extract (4.0), Pyridoxal-5-Phosphate (0.25), Tween-80 (0.5), glucose (1.5), fructose (1.0) and tyrosine (3.0) as inducer as well as substrate (Table I). The growth of the strain was measured spectrophotometrically at A_{600}. A loopful culture of *L. lactis* was inoculated in tyramine production medium and incubated at 25 °C. (150 rpm) for 8 h and used as seed. Seed culture (10%, v/v) was used as incoculum for 50 mL production medium. The amount of tyramine produced was measured by HPLC.

Quantification of tyramine by HPLC and sample preparation

Tyramine production was determined by quantification with HPLC (Perkin Elmer) equipped with a reverse phase column (Discovery HSF5 column, 0.5 µm, 5.0 cm, 4.6 mm) and UV spectrophotometer (Applied Biosystem 785 A programmable absorbance detector). The retention time for L-tyrosine and tyramine was found to be 10.58 and 20.06 min, respectively. The amount of tyramine formed was calculated from the respective standard curves (10 µg/mL 100 µg/mL). Ammonium formate (50 mM), pH –3.0 was used as mobile phase at flow rates of 1.0/mL/min Injection volume was 10 µL and detection was performed at 266 nm. Samples were prepared by centrifuging the fermentation broth (10,000 *g* for 10 min) to remove all the suspended particles. The supernatant was filtered through 0.22 µm filters and the 10 µL of the samples were loaded on the HPLC column.

Optimization of conditions for the biotransformation of L-tyrosine to tyramine by the growing cells of L. lactis

The following conditions such as selection of medium [M1, M2, M3 [27], M4 [28], M5 [29], M6 [30], M7 [31], M8 (L B medium), M9 (Nutrient Broth), M10 [32], incubation time (12, 24, 36 and 72 h), pH (4.5–10.5) and production temperature (25–55 °C) were studied for biotransformation of tyrosine to tyramine using growing cells of *L. lactis*. In each experiment final pH, growth and tyramine concentration were determined (Table I).

Table I

Selection of medium for the production of tyramine by the growing cells of *Lactococcus lactis*

Media	Media components (g/L)	Growth (mg/mL)	Tyramine concentration (μg/mL)	Final pH of broth	Reference
M1	Yeast extract 8.0, beef extract 8.0, peptone 10.0, sodium acetate 5.0, trisodium citrate 2.0, K_2HPO_4 2.0, $MgSO_4$ 0.2, $MnSO_4$ 0.1, tween-80 1.0 ml, L-tyrosine 1.0 and glucose 1.0	NG	–	5.50	[25]
M2	Meat extract 8.0, tryptone 5.0, yeast extract 4.0, l-tyrosine 3.0, glucose 1.5, fructose 1.0, tween-80 0.5, pyridoxal-5-phosphate 0.25, $MgSO_4$ 0.2, $CaCO_3$ 0.1, $MnSO_4$ 0.05 and $FeSO_4$ 0.04	10.01	6.2	6.32	[26]
M3	KH_2PO_4 13.2, $MgSO_4{\cdot}7H_2O$ 0.3, $(Na_4)3SO_4$ 1.0 and l-tyrosine 2.0	7.40	3.2	5.71	[27]
M4	Glucose 20.0, casein enzyme hydrolysate 10.0, yeast extract 5.0, phosphate buffer 0.1 M and l-tyrosine 2.0	NG	–	5.50	[28]
M5	Peptone 5.0, beef extract 5.0, yeast extract 0.5, NaCl 2.0 and l-tyrosine 2.0	5.00	0.86	5.89	[29]
M6	Meat extract 5.0, yeast extract 5.0, peptone 5.0, NaCl 2.0 and l-tyrosine 2.0	7.20	2.1	7.13	[30]
M7	Peptone 5.0, NaCl 5.0, yeast extract 10.0 and l-tyrosine 1.0	4.0	–	7.48	[31]
M8	Tryptone 10.0, yeast extract 5.0, NaCl 10.0 and l-tyrosine 1.0	6.1	1.8	8.71	LB medium
M9	Beef extract 1.0, yeast extract 2.0, peptone 5.0, NaCl 5.0 and l-tyrosine 1.0	4.4	–	8.71	Nutrient broth
M10	Peptone 5.0, yeast extract 1.0, K_2HPO_4 3.0, KH_2PO_4 0.1, $MgSO_4$ 0.5 and l-tyrosine 1.0	5.7	–	8.51	[32]

NG: No growth

Effect of substrate concentration on production medium

The L-tyrosine acts as substrate to be converted to tyramine as well as inducer for tyrosine decarboxylase. The varying concentration (0.1 0.7%) of L-tyrosine was added to the tyramine production medium.

Course of fermentation and feeding behaviour of inducer at 4, 8 and 12 h of growth for biotransformation

The growth and tyramine production profile of *L. lactis* was studied by growing the isolate in tyramine production medium. L-tyrosine at final concentration of 0.2% (w/v) was fed after 4 and 8 h of growth of *L. lactis* and periodical sampling was carried out at regular interval of 2 h after commencement of feeding. The growth, tyramine production and final pH were determined in each sample.

Results

Optimization of conditions for the biotransformation of L-tyrosine to tyramine by the growing cells of L. lactis

Selection of production medium: *L. lactis* was grown at 25 °C (150 rpm) for 16 h in ten different media. The selection of medium was done on its influence on growth and tyramine production. It was evident from Table I that the medium M2 showed the maximum production of tyramine (6.2 μg/mL) as well as growth (10.0 mg/mL). Whereas, the media M3, M6 and M8 showed comparable growth (7.4, 7.2 and 6.1 mg/mL, respectively) as well as tyramine production. The final pH was also determined in the different media. The final pH was found to be increased in each case.

Selection of incubation time: The maximum tyramine production (5.8 μg/mL) was obtained after 24 h of incubation (Fig. 1). Samples were taken at regular time intervals of 12 h and cell mass and tyramine concentration was determined. The maximum tyramine production (5.8 μg/mL) was obtained after 24 h of incubation, however, further increase in incubation time showed a 50% decrease in tyramine production has been observed at 36 h.

Optimization of pH: The influence of pH on bacterial growth and tyramine production was determined. A clear influence of pH on tyramine produc-

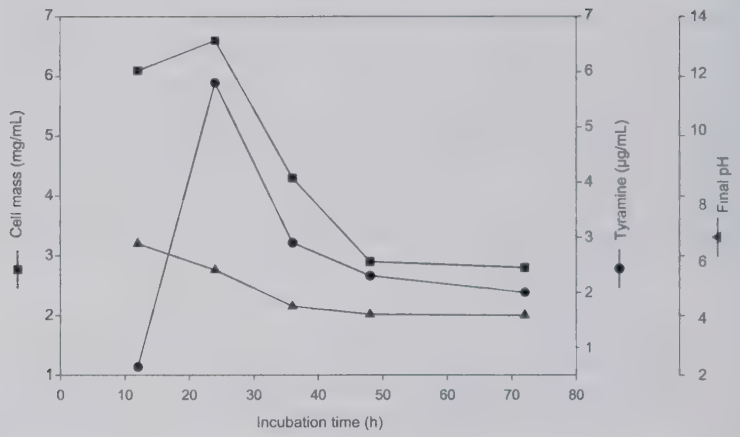


Figure 1. Effect of incubation time on tyramine production by the growing cells of *L. lactis*

tion was observed. Tyrosine decarboxylase of *L. lactis* was found to be active at the pH range of 5.0–6.5 with an optimal activity at pH 5.5. Moreover, at pH 5.5, the maximum cell mass (6.2 mg/mL) as well as maximum (6.2 µg/mL) tyramine production has been achieved (Fig. 2). However, very little growth (1.7 mg/mL) was also observed at pH 4.0. Moreover, significant growth has been observed at

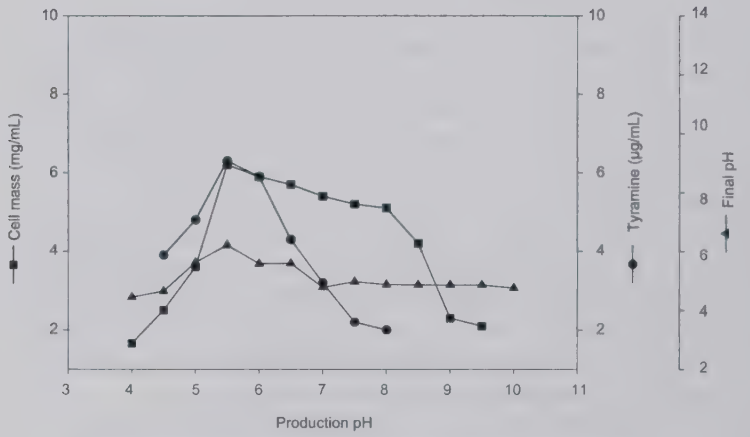


Figure 2. Effect of pH on growth and tyramine production by *L. lactis*

alkaline pH (2.1 mg/mL cell mass at pH 9.5) but tyramine synthesis could not be obtained at pH beyond 7.0. This suggests that the tyrosine decarboxylase of *L. lactis* was active only at acidic range of pH. There was a slight increase in final pH of the broth was observed with growth.

Optimization of incubation temperature: The results (Fig. 3) revealed that the isolate *L. lactis* was found a mesophilic bacteria with an optimum temperature 30 °C for growth (6.6 mg/mL) and tyramine production (10.3 µg/mL). However, with further increase in incubation temperature, a decrease in growth as well as tyramine production was observed. Moreover, no growth of the *L. lactis* has been observed at incubation temperature at and above 40 °C. The increase in final pH of the production medium was found to be associated with the growth of cells.

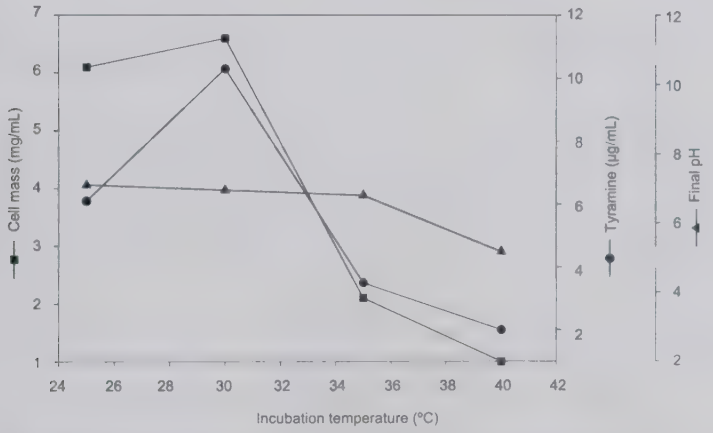


Figure 3. Effect of incubation temperature on growth and tyramine production by *L. lactis*

Optimization of L-tyrosine concentration: L-tyrosine acts as an inducer for tyrosine decarboxylase as well as substrate for tyramine biosynthesis by the *L. lactis*. In the absence of tyrosine in the medium, no tyramine was detected (Fig. 4). However, maximum tyramine was produced with 0.2% (w/v) L-tyrosine in the production medium. But with further increase in concentration of L-tyrosine, the tyramine production gradually decreased. Moreover, an insignificant decrease in cell mass was observed with increasing concentrations of L-tyrosine in the production medium. The solubility of the tyrosine beyond 0.5% (w/v) in the produc-

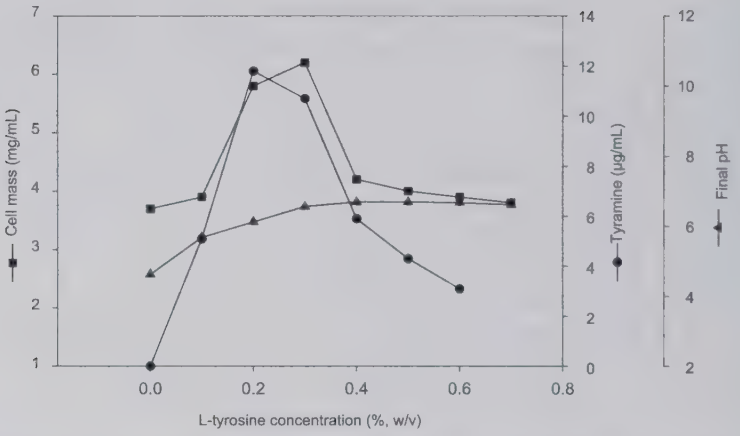


Figure 4. Effect of L-tyrosine concentration on biotransformation of L-tyrosine tyramine by growing cells of *L. lactis*

tion medium was found to be very low and the insoluble tyrosine particles interfere with the absorbance corresponding to the growth.

Course of fermentation of *L. lactis*: The course of cultivation of *L. lactis* and production of tyramine without pH control (Fig. 5). Samples were withdrawn at regular interval of 2 h and analyzed for pH, cell mass and tyramine. In the initial

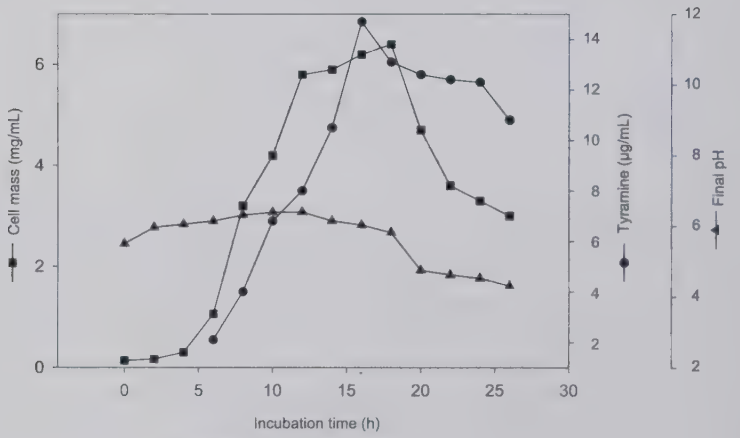


Figure 5. Course of cultivation of *L. lactis*

phase of fermentation, the active cell growth continued up to 16th h of incubation and then attained the stationary phase of growth. The maximum cell mass was found to be 6.4 mg/mL after 16th h of incubation. During the initial phase of growth, the pH of the broth increase, which at the later phase of growth decreased to a level of 4.3. This might be due to the release of more carbon dioxide due to the decarboxylation reactions at the later phase of the *L. lactis* growth. The maximum tyramine production was found to be 14.7 μg/mL at 16th h of cultivation. The fermentation time, at which the tyrosine decarboxylase activity reached its maximum value, coincided exactly with that of maximum cell growth. However, with increase in incubation time beyond 16th h, a decrease in tyramine production was observed.

Fed batch study for the biotransformation of L-tyrosine to tyramine by growing cells of L. lactis

L-tyrosine at final concentration of 0.2% (w/v) has been added to the 4 and 8 h old broth of *L. lactis* and further incubated at 30 °C for 28 h. After commencement of feeding, the tyramine concentration was determined at 2 h intervals (Table II). It was evident from the results that low tyramine production was observed in

Table II

Fed batch study for the biotransformation of L-tyrosine to tyramine by growing cells of *L. lactis*

Time (h) after commencement of feeding	Amount of tyramine (μg/mL) produced	
	Feeding to 4 h old broth	Feeding to 8 h old broth
0	–	–
2	–	–
4	–	–
6	1.83	–
8	2.88	1.31
10	3.86	1.45
12	4.95	1.62
14	5.42	2.54
16	4.32	8.21
18	4.12	5.30
20	4.01	4.65
22	3.88	4.23
24	3.80	4.01
26	3.77	3.98
28	3.70	3.89

comparison to the earlier experiments, where L-tyrosine has been added to the medium with all other components. The maximum tyramine production (8.21 μg/mL) was achieved at 16th h of feeding to the 8 h old fermentation broth. However, 5.42 μg/mL tyramine was obtained at 14th h of feeding to the 4 h old broth. There was no induction of tyrosine decarboxylase at 12 h feeding but in 4 h feeding cell growth increased from 0.35 mg/mL to 5.7 mg/mL and maximum conversion (5.42 μg/mL) was observed after 16 h. Whereas in case of 8 h feeding cell growth increased from 0.8 mg/mL to 6.6 mg/mL and tyramine concentration 8.2 μg/mL.

Discussion

The tyramine production by *L. lactis* was improved further by optimizing the production conditions. Selection of production medium has been carried out on the basis of growth and tyramine production. The selected production medium contained (g/L) meat extract 8.0, tryptone 5.0, yeast extract 4.0, L-tyrosine 2.0, glucose 1.5, fructose 1.0, tween-80 0.5, Pyridoxal-5-PO_4 0.25, $MgSO_4$ 0.2, $CaCO_3$ 0.1, $MnSO_4$ 0.05 and $FeSO_4$ 0.04. In this medium there was no interference due to the acidification caused by the fermentation of sugars, however, some added glucose and fructose help in better growth of microorganism. The low glucose concentration, low pH (5.5) and the presence of pyridoxal-5-Phosphate in the medium have a strong enhancing effect of amino acid decarboxylase activity [12, 13]. Other added components include the cations and metal ions, which also support the decarboxylase activity.

The growth requirements for the decarboxylase production by *S. faecalis* were found to be more specific than the requirements for cell growth [14]. In their study, they found a medium of essentially known composition was most suitable for the production of *S. faecalis* cells with tyrosine decarboxylase activity. Other factors influencing the decarboxylase production had also been studied and confirmed the adaptive nature of tyrosine decarboxylase. In the present study tyrosine decarboxylase of *L. lactis* has been found to be pyridoxal dependent as it is clear from the results obtained in the selection of media in which the medium with pyridoxal-5-phosphate showed maximum tyramine production. In another study the *S. faecalis* cells were grown in media deficient in pyridoxine derivatives have been shown to contain the tyrosine decarboxylating enzyme but not the coenzyme. Pyridoxal was found to be important coenzyme for tyrosine decarboxylase enzyme [13]. However, pyridoxal requires the multivalent metal ion. A requirement

for Fe^{3+} ions was also reported but copper ions were found to inhibit the bacterial amino acid decarboxylase [15]. The pH level was found to be a key factor in influencing the amino acid decarboxylase activity. In the present study, maximum conversion of L-tyrosine to tyramine by *L. lactis* was observed at acidic pH (5.5). Similar results have also been reported earlier [16, 17]. The influence of initial pH of the medium on tyramine production was described for *E. faecium* and *L. brevis* [18]. During the initial phase of growth of *L. lactis*, a slight increase in pH has been observed, however, at the later stage of fermentation, the pH started decreasing. It has been observed that reduction in the culture pH simply reduced the growth rate and in turn, tyramine production by *E. faecalis* [19]. Fernandez et al. [11] have also studied the factors affecting the tyramine production by *E. durans* IPLA 655 and found lesser tyrosine decarboxylase activity in the medium with pH 6.8. However, Anderson et al. [20] used a medium of pH 7.0 for production of tyrosine decarboxylase by *S. faecalis*. The interesting point was the fact that the optimum pH for the medium for production of tyrosine decarboxylase by *S. faecalis* was 5.5. They noted that *S. faecalis* retained 90% of its tyrosine decarboxylase activity at pH 7.0. Therefore, it was possible to use this enzyme along with tyrosine phenol lyase (optimum pH 7.0) in a multienzyme bioreactor for the biosynthesis of tyrosine from phenol. *L. lactis* was found to be mesophilic bacteria, with an optimum temperature 30 °C for growth and tyramine production. Moreover, no growth was detected beyond 35 °C. Several authors have also reported that the decarboxylase activity depends on temperature and increases with incubation time and storage temperature [19, 21]. Maximum conversion of L-tyrosine to tyramine by the growing cells of *L. lactis* has been obtained at 16^{th} h of incubation. In a similar study, the *L. brevis, L. casei, L. collinoides, L. paracasei* and *L. plantarum* isolated from wine samples were found to produce tyramine at mid-log phase of their growth [22]. L-tyrosine acts as an inducer for tyrosine decarboxylase as well as substrate for tyramine biosynthesis for the *L. lactis*. L-tyrosine i nduction was also found to play important role in tyrosine decarboxylase biosynthesis [23, 24] and hence, 2.0 g/L L-tyrosine has been added to the culture medium. Moreno-Arribas and Funel [25] showed that the tyrosine decarboxylase activity of *L. brevis* depend on the availability of L-tyrosine in the culture medium. L-tyrosine at a concentration of 2.0 g/L in the production medium was found to be most suitable for the biotransformation of L-tyrosine to tyramine. However, better biotransformation was achieved when L-tyrosine was added to the medium along with all other ingredients. Feeding of L-tyrosine to the pre-grown cells of *L. lactis* has also been carried out, but the tyramine level was found to be on lower side. Moreover, the

L-tyrosine insolubility at concentration beyond 5 g/L interfered with growth measurements.

The results obtained in present study provide a platform for the development of a bench level bioprocess, which could be used for the biosynthesis of commercially important molecule and intermediate.

Acknowledgements

We acknowledge the financial support in term of Junior Research Fellowship from Department of Science and Technology, Govt. of India to Miss. Meenu Thakur and we would also like to thank Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, India. The authors are thankful to Sub distributed information centre of Himachal Pradesh University for providing assistance in the data retrieval.

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SIGILLUM: ACTA MICROBIOL. ET IMMUNOL. HUNG.
CODEN: AMIHEC

ISSN 1217-8950